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Name of Candidate: Reyda González-Nieves
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DISSERTATION AND ABSTRACT APPROVED:

DATE:

6/8/12

Dr. Johnan Kaleeba
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
Committee Chairperson

6/8/12

Dr. Mary Lou Cutler
DEPARTMENT OF MOLECULAR AND CELL BIOLOGY
Dissertation Advisor

6/8/12

Dr. Thomas Darling
DEPARTMENT OF DERMATOLOGY, ANATOMY, PHYSIOLOGY, AND GENETICS
Committee Member

6/8/12

Dr. Saibal Dey
DEPARTMENT OF BIOCHEMISTRY
Committee Member

6/8/12

Dr. Dennis McDaniel
DEPARTMENT OF BIOMEDICAL INSTRUMENTATION CENTER, MICROSCOPIC IMAGING

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A handwritten signature in black ink, appearing to read 'R. González-Nieves', with a stylized, flowing script.

Reyda P. González-Nieves
Molecular and Cell Biology Program
Department of Pathology
Uniformed Services University
May 25, 2012

ABSTRACT

Title of Thesis/Dissertation: **“Regulation of adhesion and migration by the Rsu1- and PINCH1- mediated inhibition of focal adhesion formation and actin polymerization.”**

Author: Reyda Paola González-Nieves, Ph.D., 2012
Uniformed Services University of the Health Sciences

Thesis directed by: Mary Lou Cutler, Ph.D., Professor
Department of Pathology
Uniformed Services University of the Health Sciences
4301 Jones Bridge Road, Bethesda, Maryland 20814

The Rsu1-ILK-PINCH-Parvin (RIPP) complex functions as an adaptor between integrins and the actin cytoskeleton and contributes to the regulation of adhesion and migration. The IPP focal adhesion (FA) complex is composed of the proteins **ILK**, **PINCH1** and **Parvin** as well as an associated protein Rsu1, which binds to the LIM 5 domain of the adaptor protein PINCH1. Depletion of Rsu1 or PINCH1 inhibits mammary epithelial cell adhesion and migration. While Rsu1 binding to PINCH1 and the complex is associated with processes such as cell adhesion and migration, the exact mechanism by which this occurs remains to be elucidated. The present work investigates the mechanistic role of Rsu1 in IPP signaling. Our data demonstrated the effects of Rsu1 and PINCH1 depletion on adhesion, migration, FA formation and actin cytoarchitecture in MCF-10A cells.

An siRNA-mediated approach was used to examine the effects of Rsu1 or PINCH1 depletion on adhesion and migration in a non-tumorigenic mammary epithelial cell line, MCF-10A. The depletion of Rsu1 or PINCH1 by siRNA in MCF-10A cells results in a change in the distribution and localization of the FA proteins β 1 integrin, vinculin, talin, paxillin and ILK. Control cells showed a peripheral and highly organized staining of FA proteins, while the Rsu1- or PINCH1-depleted cells exhibited disorganized staining throughout the cell body. Western blotting revealed that the absolute level of many FA proteins did not change significantly in response to Rsu1 or PINCH1 knockdown. However, cells depleted of Rsu1 or PINCH1 displayed elevated levels of β 1 integrin and decreased EGF-R. Also, there was a decrease in FAK phosphorylation in Rsu1 and PINCH1 depleted cells that correlated with the inability of the cells to create new FA sites. Rsu1 or PINCH1 depleted cells adhered poorly to substrate, displayed a dramatic decrease in migration, exhibited disorganized caveoli and retained the ability to activate Rac in response to EGF.

Examination of actin cytoarchitecture by confocal microscopy showed the loss of stress fibers and the formation of actin rich structures at the cell periphery in Rsu1 or PINCH1 depleted cells. Phosphorylation of the actin regulatory proteins VASP and cofilin occurs following Rsu1 or PINCH1 depletion thereby blocking proper actin polymerization. Depletion of Rsu1 caused a substantial reduction in PINCH1, but PINCH1 depletion resulted in only a modest reduction in Rsu1. This implies a function for Rsu1 in PINCH1 stability. However, reconstitution of Rsu1 depleted cells with a mutant that fails to bind PINCH1

restored actin stress fibers, FAs and migration, suggesting an Rsu1 function independent from the IPP complex in the regulation of actin-dependent adhesion and migration. Elevation of cAMP levels in Rsu1 depleted cells highlighted the contribution of the RIPP complex to cAMP, PKA and EPAC signaling. Inhibition of PKA or activation of EPAC resulted in the rescue of FAs and migration in Rsu1 depleted cells.

**Regulation of adhesion and migration by the Rsu1- and PINCH1-mediated
inhibition of focal adhesion formation and actin polymerization.**

By

Reyda Paola González-Nieves

Dissertation submitted to the Faculty of the Molecular and Cell Biology Program
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List of Abbreviations

<u>AC</u> : Adenyl Cyclase	<u>GTP</u> : Guanosine Triphosphate
<u>ANK</u> : Ankyrin	<u>ILK</u> : Integrin Linked Kinase
<u>ATP</u> : Adenosine Triphosphate	<u>IPP</u> : ILK-PINCH-Parvin
<u>BSA</u> : Bovine Serum Albumin	<u>NK</u> : c-Jun-N-terminal Kinase
<u>cAMP</u> : Cyclic Adenosine Monophosphate	<u>kDa</u> : Kilodalton
<u>cDNA</u> : Complementary DNA	<u>Limk1</u> : Lim kinase1
<u>cGMP</u> : Cyclic Guanosine Monophosphate	<u>LRR</u> : Leucine Reach Repeat
<u>DAPI</u> : 4,6' diamidino-2-phenylindole	<u>nm</u> : nanometer
<u>ECM</u> : Extracellular Matrix	<u>OVN</u> : overnight
<u>EEA1</u> : Early Endosome Marker	<u>PBS</u> : Phosphate Buffered Saline
<u>EGF</u> : Epidermal Growth Factor	<u>PDE</u> : Phosphodiesterase
<u>EGFR</u> : Epidermal Growth Factor Receptor	<u>PH</u> : Plekstrin Homology
<u>ELISA</u> : Enzyme-Linked Immunosorbent Assay	<u>PI3K</u> : Phosphoinosilide 3-Kinase
<u>EPAC</u> : Exchange Protein Activated by cAMP	<u>PKA</u> : Protein Kinase A
<u>FA</u> : Focal Adhesion	<u>PKC</u> : Protein Kinase C
<u>FAK</u> : Focal Adhesion Kinase	<u>PLP</u> : Paraformaldehyde
<u>FITC</u> : Fluorescein Isothiocyanate	<u>PP1α</u> : Protein Phosphatase 1 alpha
<u>GPCR</u> : G Protein Coupled Receptors	<u>PTP</u> : Protein Tyrosine Phosphatase

RACK1: Receptor for Activated Protein Kinase C

RIAM: Rap1 GTP-interacting adaptor molecule

RIPP: Rsu1-ILK-PINCH-Parvin

RNA: Ribonucleic Acid

Rsu1: Ras Suppressor Protein 1

RT: Room Temperature

RTK: Receptor Tyrosine Kinase

RT-PCR: Reverse Transcriptase Polymerase Chain Reaction

SE: Standard Error

Ser: Serine

siRNA: small interfering RNA

Tyr: Tyrosine

UTR: Untranslated Region

VASP: Vasodilator-Stimulated Phosphoprotein

Chapter 1: Dissertation Introduction

Overview

This thesis work investigated the mechanism by which cell adhesion and migration are regulated in mammary epithelial cells by the Rsu1-ILK-PINCH-Parvin (RIPP) complex of proteins. The evidence from many groups established the RIPP as a requirement for cell adhesion and migration; however, knowledge of the mechanisms underlying this regulation remains incomplete. The experiments presented here were conducted in a non-tumorigenic mammary epithelial cell line (MCF-10A). MCF-10A cells lack anchorage-independent growth and depend on growth factors and hormones for proliferation and survival. Most importantly, MCF-10A cells have the ability to be grown in three dimensional cultures and migrate in response to the epidermal growth factor (EGF). An siRNA mediated approach was used to examine the effects of Rsu1 or PINCH1 depletion in cell adhesion, migration, focal adhesion (FA) formation and the actin cytoskeleton cytoarchitecture.

The extracellular matrix

The extracellular matrix (ECM) is a highly conserved non cellular component composed of an insoluble network of proteins and polysaccharides that provides structural support to cells (Frantz et al., 2010; Geiger and Yamada, 2011; Hynes, 2009). Cells adherent to the matrix are responsible for the production of the macromolecules that constitute the ECM and the control of the orientation of the matrix (Geiger et al., 2001). In addition to its scaffolding properties, the ECM is considered a dynamic structure that generates and transmits signals that control cell behavior (Boudreau and Jones, 1999; Hynes, 2009). Other major functions of the matrix include effects on cell polarity, cell

adhesion, cell survival, proliferation, differentiation, tissue regeneration and cell migration (Frantz et al., 2010; Hynes, 2009).

The ECM is classified in two main forms: the interstitial matrix and the basement membrane (also referred to as the basal lamina) (Frantz et al., 2010). The interstitial matrix is composed of the ECM located in connective tissue such as bone, tendon and the dermal layer of the skin. In epithelial tissues, the ECM forms a small, flexible, tough, thin layer of matrix molecules that underlies the epithelia called the basement membrane. This basal lamina is synthesized by cells in the epithelial layer and the connective tissue (called the stroma). Its two main components are fibrous proteins (usually glycoproteins) and glycosaminoglycans. Even though the composition of the basal lamina differs based on tissue type, it usually contains glycoprotein laminins, type IV collagen, nidogen, and the proteoglycan perlecan. The two forms of ECM differ in their properties and composition based on physical location within and developmental stage of the organism (Frantz et al., 2010). Deregulation in the formation of the ECM or one of its components is associated with a variety of genetic defects and diseases, including tumor invasion and metastasis (Jarvelainen et al., 2009).

Cells control the synthesis, organization and degradation of the ECM. The ECM exerts a critical function in the architecture and behavior of cells. Cells will receive signals from the ECM through transmembrane cell adhesion proteins called matrix receptors. A number of molecules have the ability to act as matrix receptors or co-receptors (proteoglycans), but integrins are the main family of proteins that mediate the interaction of cells with the extracellular matrix (Arnaout et al., 2007; Barczyk et al., 2010; Cabodi et al., 2010; Hynes, 2002; Hynes, 2004; Tamkun et al., 1986). One of the

remarkable characteristics of the integrins is the ability to perform “bidirectional signaling” (Arnaout et al., 2005; Coppelino and Dedhar, 2000; Hynes, 2002); they can receive signals from the ECM and transmit it to the cell as well as relay signals from the inside of the cells back to the ECM.

Integrins

Integrins are heterodimeric type I transmembrane glycoproteins composed of α (alpha) and β (beta) subunits (Berman and Kozlova, 2000; Berman et al., 2003; Takada et al., 2007). There are 24 combinations of integrins in humans. The extracellular portion of the integrins binds to extracellular matrix proteins (such as fibronectin), matrix proteases, growth factors receptors, and other receptors (Miranti and Brugge, 2002). The internal portion of the integrins associates with actin cytoskeletal and signaling proteins (Barczyk et al., 2010; Hynes, 2002; Longhurst and Jennings, 1998; Takada et al., 2007). There is a body of evidence that implicates the cytoplasmic domains of the α and β subunits in integrin activation (Barczyk et al., 2010; Hynes, 2002; Longhurst and Jennings, 1998; Takada et al., 2007). In an inactive or low-affinity state, the extracellular domain of the integrins is in a “bent” conformation, which is characterized by a masked ECM binding pocket (Legate et al., 2006). Binding of talin, filamin, migfilin, FAK or ILK to the cytoplasmic domain of integrins results in their activation (Calderwood, 2004; Ithychanda et al., 2009; Kiema et al., 2006; Michael et al., 2009; Tadokoro et al., 2003). Once the integrins are activated, their extracellular domains switch to an open conformation, permitting an association with the ECM and growth factor receptors (Giancotti, 2003; Giancotti and Ruoslahti, 1999). Binding to the ECM initiates a signaling cascade that results in the creation of focal adhesions (FAs).

FAs are integrin-adhesion sites characterized by the association of integrin clusters with the actin cytoskeleton and numerous adhesion and signaling molecules. Over 100 proteins can be recruited to these adhesion sites (Zamir and Geiger, 2001). In adherent cells, the FAs are stable. In contrast, motile cells have a constant formation and dissolution of FAs resulting in the migration of cells. The assembly and disassembly of FAs are required for both cell adhesion and migration (Geiger et al., 2009; Lo, 2006; Parsons et al., 2010; Wozniak et al., 2004).

The RIPP complex

Integrins play fundamental roles in the processes of adhesion, spreading and migration (Chan et al., 2007; Giancotti, 2003; Giancotti and Ruoslahti, 1999; Schatzmann et al., 2003). Consequently, altered integrin signaling has been associated with tumor progression, migration and invasion. (Chan et al., 2007; Guo and Giancotti, 2004; Schatzmann et al., 2003). Several studies have identified various proteins (Cabodi et al., 2010; Legate et al., 2006) that are important regulators of integrin activity, including the integrin linked-kinase (ILK), the adaptor protein PINCH, Parvin and Rsl1 (**Figure 1**) (Legate et al., 2006; Wickstrom et al., 2010b; Wu, 2004). These proteins form a complex called the RIPP complex, which is located in the cytoplasm of the cells; however, upon integrin activation, this complex is recruited to the integrin-containing focal adhesions (FAs). Interestingly, the stability of the RIPP complex is dependent upon the expression of each of the individual components (Fukuda et al., 2003). The absence of a member of the complex results in the degradation of the other components (Legate et al., 2006; Zhang et al., 2002a).

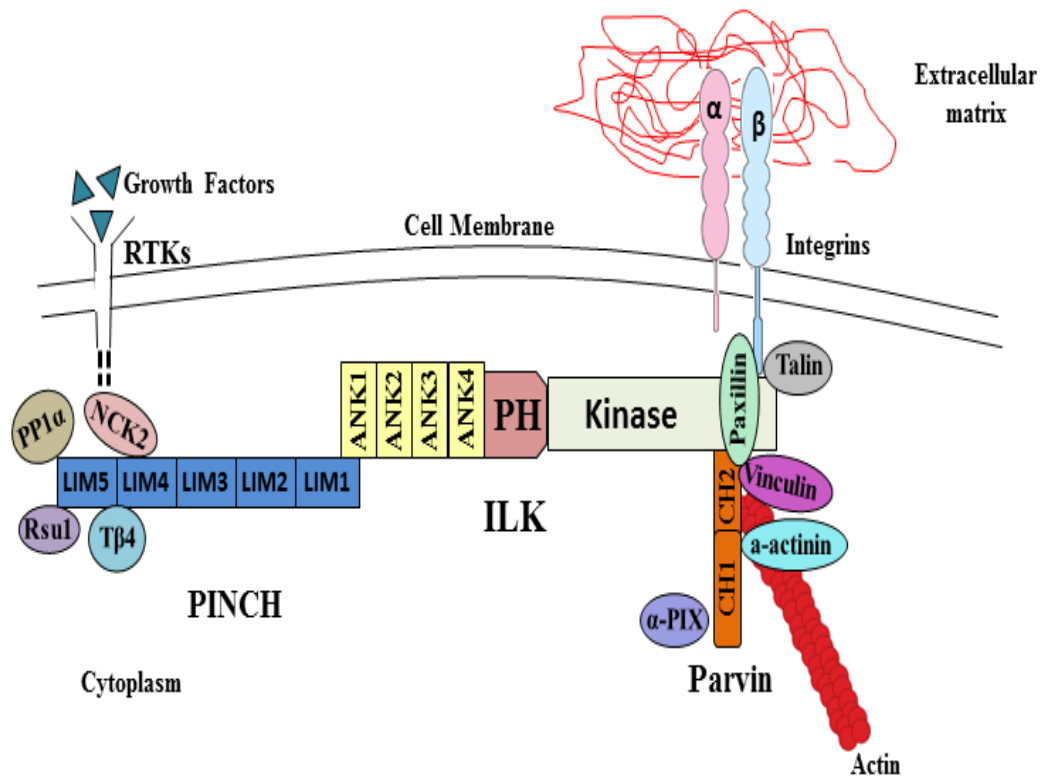


Figure 1. The RIPP complex. The RIPP (Rsu1-ILK-PINCH1-Parvin) complex is located in the cytoplasm of the cells, but upon integrin activation, this complex is recruited to the integrin-containing focal adhesions (FAs). ILK is the central member of the IPP complex; it binds directly to the intracellular tail of β integrins. It also interacts with focal adhesion (FA) proteins (Talin, Vinculin, Paxillin) and the actin cytoskeleton via Parvin. PINCH1 adaptor protein binds directly to the N terminal region of ILK and connects the RIPP complex to growth factor signaling through the binding of NCK2 to receptor tyrosine kinases (RTKs). PINCH1 associates with proteins that include Rsu1, thymosin β 4 (T β 4) and PP1 α influencing Jun N-terminal kinase signaling, cell migration and survival. The Ras suppressor protein1 (Rsu1) functions as part of the IPP complex via binding to the LIM5 domain of the adaptor protein PINCH1. Parvin binds to the pseudokinase domain of ILK; it interacts directly and indirectly with F-actin and the guanine nucleotide factor α -PIX.

ILK is the central member of the IPP complex and consists of three different domains: N-terminal ankyrin (ANK) repeats, a plekstrin homology (PH) domain and a C-terminal pseudokinase domain. The ANK1 domain binds to the LIM1 domain of the adaptor protein PINCH. A LIM domain is a cysteine-rich zinc-binding motif associated with protein-protein interactions (Legate et al., 2006). The pseudokinase domain binds to parvins, paxillin and the cytoplasmic domain of β integrins. PINCH, which is a LIM domain protein, hypothetically connects integrin signaling to growth factor signaling through the binding of NCK2 to receptor tyrosine kinases (RTKs). Additionally, PINCH1 associates with proteins that include Rsu1, β thymosin and PP1 α , thereby influencing Jun N-terminal kinase signaling, cell migration and survival. Parvin associates with the pseudokinase domain of ILK and interacts directly or indirectly with F-actin and proteins that are involved in cytoskeleton remodeling such as paxillin, α -actinin or α -PIX (Legate et al., 2006).

Within the focal adhesions, the RIPP complex functions as an adaptor between integrins and the actin cytoskeleton and also as a center for signaling networks (Cabodi et al., 2010; Legate et al., 2006). Additionally, the complex plays a role in integrin activation (McDonald et al., 2008), actin cytoskeleton remodeling (Li et al., 2005; Sakai et al., 2003) and FA formation (Sakai et al., 2003; Stanchi et al., 2009). These functions are of interest in cancer because changes in Rsu1, PINCH1 or ILK result in altered cell adhesion, spreading and motility in mammalian cells (Dougherty et al., 2005; Simpson et al., 2008; Tu et al., 1999; Zhang et al., 2002b). Importantly, the levels of ILK and PINCH1 increase in common solid tumors and reactive stroma (Hannigan et al., 2005; Wang-Rodriguez et al., 2002). In contrast, Rsu1 is alternatively spliced, resulting in the

production of an inactive form of the protein in high grade tumors and tumor cell lines (Chunduru et al., 2002; Dougherty et al., 2008).

Rsu1 is an ubiquitously expressed single-copy gene in mammalian and other eukaryotic cells (Cutler et al., 1992; Dougherty et al., 2008; Kadrmas et al., 2004; Sieburth et al., 1998). An important finding from our laboratory is that Rsu1 functions as part of the IPP complex via binding to the LIM5 domain of the adaptor protein PINCH1 (Dougherty et al., 2005). Rsu1 colocalizes with PINCH1 and ILK at FA sites in mammalian cells (Dougherty et al., 2008). One of the hallmarks of this 33 kDa protein is the ability to suppress Ras transformation (Cutler et al., 1992). Previous studies demonstrated that ectopic expression of Rsu1 cDNA not only prevented Ras transformation but also inhibited anchorage independent growth in various human tumor cell lines including breast cancer cells (Cutler et al., 1992; Tsuda et al., 1995; Vasaturo et al., 2000).

RT-PCR and sequencing of the Rsu1 open reading frame revealed that exon 8 is missing in the alternatively spliced Rsu1 RNA in human gliomas and breast tumors, as well as in many tumor cell lines (Chunduru et al., 2002; Dougherty et al., 2008). Dougherty *et al.* demonstrated that the exon 8-deleted 29 kDa Rsu1 protein is expressed in response to activation of the Ras-MEK-ERK signal and does not bind PINCH (Dougherty et al., 2008). Ectopic expression of full length Rsu1 resulted in an increase in cell spreading, more abundant actin structures and a decrease in JNK and Rac activation (Dougherty et al., 2008; Masuelli and Cutler, 1996). The Rac GTPase is actively involved in the process of cell migration by promoting actin polymerization at the leading edge of the cell (Yamazaki et al., 2005). Conversely, the depletion of the full length Rsu1

transcript correlated with increased cell migration, JNK and Rac activation (Dougherty et al., 2008). While Rsu1 levels may regulate Jun kinase activity (Dougherty et al., 2005; Masuelli and Cutler, 1996), and hence affect Rac activation, the functions of Rsu1 and the IPP complex during migration are not completely known.

Studies performed by other groups have also revealed a role for the Rsu1 protein within the context of integrin-dependent adhesion (Kadmas et al., 2004; Simpson et al., 2008). Rsu1 protein binds and colocalizes with PINCH1 in *Drosophila*, and Rsu1 depletion or mutation produces flies with wing blisters supporting the idea that Rsu1 plays a role in cell adhesion (Kadmas et al., 2004). An siRNA screen for proteins required for cell migration revealed that depletion of Rsu1 or PINCH1 in a non-tumorigenic mammary epithelial cell line (MCF-10A) blocked EGF-dependent migration (Simpson et al., 2008; Winograd-Katz et al., 2009).

cAMP signaling pathway

cAMP signaling plays a pivotal role in cell adhesion and migration (**Figure 2**). G Protein Coupled Receptors (GPCRs) are a family of integral membrane proteins that promote the synthesis of cAMP (Lappano and Maggiolini, 2011; Neves et al., 2002). GPCRs are activated by hormones, neurotransmitters, lipids or catecholamines. Activation of GPCRs results in a conformational change that leads to the release of Gs

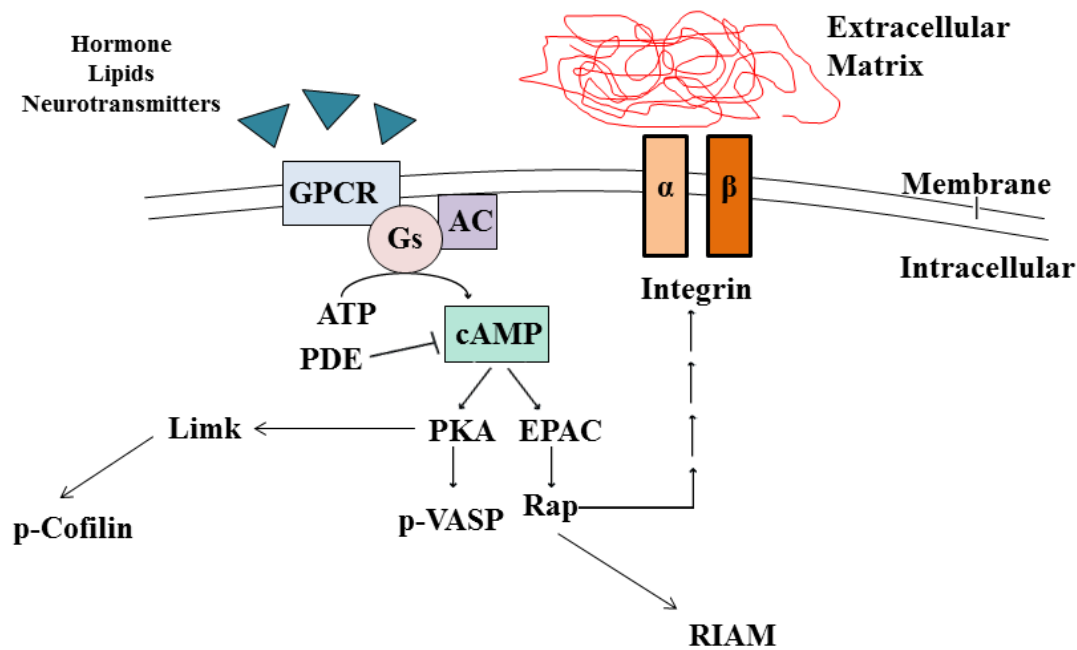


Figure 2. cAMP signaling. Activation of GPCR by hormones, lipids or neurotransmitters causes the release of the Gs protein from the G protein complex. Activation of adenylyl cyclase by Gs results in an increase in cAMP synthesis. cAMP synthesis is blocked by the actions of phosphodiesterases (PDE). cAMP activates its downstream effectors: PKA and EPAC. PKA regulates the phosphorylation of VASP and cofilin. EPAC activates Rap GTPase which leads to the activation of RIAM. Rap also plays a role in integrin activation.

alpha subunit from the G protein complex. Consequently, Gs alpha activates adenylyl cyclase (AC) which results in synthesis of cAMP (Cooper, 2003; Fimia and Sassone-Corsi, 2001; Schwartz, 2001). cAMP activates Protein Kinase A (PKA) and the Exchange Protein Activated by cAMP (EPAC) (Cheng et al., 2008; de Rooij et al., 1998). These two proteins are required for adhesion, migration, FA formation, integrin activation, actin cytoskeleton remodeling and endocytosis (Bos, 2006; Cheng et al., 2008; de Rooij et al., 1998; Gloerich and Bos, 2010; Han and Rubin, 1996; Howe, 2004; Razani et al., 1999; Whittard and Akiyama, 2001).

PKA

PKA regulates remodeling of the actin cytoskeleton in part by the phosphorylation of actin binding proteins. Vasodilator-Stimulated Phosphoprotein (VASP) is a PKA substrate that plays a role in actin cytoskeleton dynamics, adhesion and migration (Howe, 2004; Reinhard et al., 2001). VASP contributes to actin polymerization by nucleating actin filaments and providing pools of Profilin-G-actin which results in a continuous polymerization (Eckert and Jones, 2007). VASP is phosphorylated by PKA at Ser 157 (favored site) and Ser 239 (Butt et al., 1994; Eckert and Jones, 2007; Smolenski et al., 1998), disrupting the ability of this molecule to nucleate actin filaments (Walders-Harbeck et al., 2002). Additionally, PKA regulates the phosphorylation of cofilin in an indirect way (Nadella et al., 2009). Cofilin is an actin binding protein that is required for actin depolymerization and severing of actin filaments (Moriyama et al., 1996; Moriyama and Yahara, 2002). Phosphorylation of cofilin by LIM kinase 1 (Limk1), results in the inhibition of cofilin (Heredia et al., 2006). Hence, PKA does not directly interact with cofilin but regulates its function via Limk1 (Nadella et al., 2009).

EPAC

EPAC is a Rap guanine nucleotide exchange factor (Bos, 2006; Breckler et al., 2011). Rap is a member of the Ras family of GTPases (Bos, 2005; Gloerich and Bos, 2010). Rap cycles from an active (GTP-bound) to inactive conformation (GDP-bound) (Bos, 2005) and controls cell adhesion by the regulation of integrin affinity and clustering (Bos, 2005). A mechanism by which Rap1 controls integrin cell adhesion and spreading is by the regulation of RIAM or lamellipodin. Depletion of RIAM in Jurkat T cells disrupts Rap1 induced mediated adhesion (Bos, 2005). Additionally, RIAM interacts with

Profilin and VASP demonstrating a role for RIAM in actin dynamics (Lafuente et al., 2004).

A decrease in cell adhesion correlates with elevated levels of cAMP (Norambuena and Schwartz, 2011). Norambuena *et al.* demonstrated that loss of proteins from lipid rafts causes an increase in cAMP (Norambuena and Schwartz, 2011). While many raft components are internalized following cell detachment, G α s remains in the plasma membrane (Norambuena and Schwartz, 2011). However, loss of G α s from the lipid rafts results in a more direct interaction with AC leading to an increase in the number of activated G α s available to activate AC and subsequently an increase in cAMP production (Norambuena and Schwartz, 2011).

The FAK, RACK1 and PDE4D5 complex

Serrells *et al.* demonstrated that the elevation of cAMP levels upon cell detachment was a result of destabilization of the FAK1, RACK1 and PDE4D5 complex. This complex is recruited to nascent adhesions maintaining low levels of cAMP (Serrells et al., 2011; Serrells et al., 2010).

FAK

FAK is a signaling molecule that plays crucial roles in cell spreading, survival, proliferation and migration (Dumbauld et al., 2010; Petit and Thiery, 2000; Serrells et al., 2007). The activated form of FAK, p-FAK, is essential in FA signaling and turnover (Cary et al., 1996; Ilic et al., 1995). FAK is activated by integrin and growth factor signaling (Mitra et al., 2005; Parsons, 2003). The best characterized event in FAK phosphorylation is its autophosphorylation at Tyr 397 (Toutant et al., 2002). Phosphorylation at Tyr 397 results in the formation of phosphotyrosine docking sites that

leads to the recruitment and activation of proteins such as Src family kinases, phospholipase C γ , SOCS, Grb-7, Shc, p120 RasGAP, and the p85 subunit of phosphatidylinositol 3-kinase (Mitra et al., 2005; Parsons, 2003). Activation of these proteins is involved in the phosphorylation of other phosphorylation sites in FAK. Protein tyrosine phosphatases (PTP's) also regulate the activity of FAK in a positive or negative manner (Chiarugi et al., 2003; Zeng et al., 2003). Serrels *et al.* identified the FAK-FERM domain (residues E139 and D140) as the domain responsible for RACK1 binding (Serrels et al., 2010). FERM domains are protein modules usually located at the N-terminal region of proteins. They typically bind phospholipids, membrane and cytoskeletal proteins (Frame et al., 2010).

RACK1

RACK1 was first identified as a receptor for protein kinase C (Ron et al., 1994). This highly conserved scaffolding protein regulates processes such as cell adhesion, migration, cytokinesis and protein translation (Serrels et al., 2011). Additionally, an interaction between RACK1 and Kindlin 3 was reported, suggesting a role for RACK1 in integrin signaling (Feng et al., 2012). Some of RACK1 binding partners include the Src family of non-receptor protein tyrosine kinases (Src and Fyn), transmembrane receptors (Insulin-like Growth Factor Receptor I, β -integrin receptor, inositol 1,4,5-trisphosphate receptors), receptor tyrosine kinases, FAK and PDE4D5 (Adams et al., 2011). Furthermore, it was established that FAK, RACK1 and PDE4D5 form a complex together and that RACK1 is likely connecting FAK and PDE4D5 (Serrels et al., 2010).

Phosphodiesterase

cAMP levels are negatively regulated by the action of phosphodiesterases (PDEs) (Fimia and Sassone-Corsi, 2001; Omori and Kotera, 2007). PDE is an enzyme that breaks the phosphodiester bond in the second messenger cAMP or cyclic guanosine monophosphate (cGMP). 11 families of PDEs have been identified (Omori and Kotera, 2007). However, the families of PDE4, PDE7, and PDE8 are specific for cAMP (Conti and Jin, 1999; Soderling and Beavo, 2000). PDE4D5 interacts with RACK1 via its N terminal region (Bolger et al., 2006; Smith et al., 2007; Yarwood et al., 1999). Serrells *et al.* demonstrated that PDE4D5 also interacts with FAK (Serrells et al., 2010).

The FAK1-RACK1-PDE4D5 complex likely exerts its function through the regulation of Rap1 via EPAC (Serrells et al., 2011). The expression of a FAK mutant that does not bind to RACK1 resulted in higher levels of Rap1 leading to impaired polarization (Serrells et al., 2011). This complex maintains low levels of Rap1 until nascent adhesion stabilization or polarization takes place (Serrells et al., 2011).

Main observations of this project

Our laboratory is interested in examining the intricate regulation of cell adhesion and migration controlled by a complex located at the focal adhesions. The research described herein investigates how FA formation and cell migration are regulated in mammary epithelial cells by the RIPP complex of proteins. Our studies revealed the effects of Rsu1 or PINCH1 depletion on adhesion, migration, FA formation and actin cytoarchitecture. Additionally, we demonstrated a function for Rsu1 that is independent of its interaction with the IPP complex. Further research revealed that the cAMP pathway is a requirement for Rsu1-regulated migration, and we identified PKA and EPAC as

important regulators of FA formation and cell migration. Furthermore, we demonstrated a requirement for Rsl1 in the localization of the RACK1-PDE4D5 complex.

Chapter 2: Experimental Methods

Cell lines

The human immortalized mammary epithelial cell line (MCF-10A) and 293T cells used in this study were obtained from the American Type Culture Collection (Manassas, VA). MCF-10A cells were maintained as described previously (Morrison et al., 2010). The 293T cell line was cultured in DMEM-low glucose supplemented with penicillin/streptomycin, glutamine and 10% fetal bovine serum. The immortalized human astrocytes (E6/E7/hTERT) and the Ras-transformed version (E6/E7/hTERT/Ras) were a generous gift from Dr. Russell Pieper. E6/E7/hTERT and E6/E7/hTERT/Ras were maintained as described (Sonoda et al., 2001a; Sonoda et al., 2001b).

siRNA

Rsu1 or PINCH1 depletions were accomplished using siRNA-mediated inhibition of gene expression in a reverse transfection protocol as previously described (Dougherty et al., 2008). The sequences of the siRNA's (Thermo Fisher Scientific, Lafayette, CO) targeting Rsu1 and PINCH1 are: Rsu1: 5'-GGGAUAACGACCUGAUCUCUU-3', Rsu1 (UTR): 5'-GAACAAAGCUCU UAUUCAAUU-3' and PINCH1: 5'-UGGUCUCUGCUCUUAA UAAAdTdT-3'. The control siRNA used in this study is a standard negative control siRNA (Qiagen, Valencia, CA). The siRNAs were used at a concentration of 75 nM. Cells were collected 72-96 hours post-transfection.

Western blotting

Cell lysates were collected in RIPA buffer and processed as described previously (Dougherty et al., 2005) (Galbaugh et al., 2006). The antibodies used in this study include mouse anti-talin (Sigma-Aldrich, St. Louis, MO), mouse anti-paxillin (BD Transduction, BD Biosciences, San Diego, CA), mouse anti-vinculin (Sigma-Aldrich, St. Louis, MO), mouse anti-caveolin (BD Transduction, BD Biosciences, San Diego, CA), mouse anti- β -actin (Sigma-Aldrich, St. Louis, MO), rabbit anti-actopaxin/parvin (Sigma-Aldrich, St. Louis, MO), mouse anti-FAK (BD Transduction, BD Biosciences, San Diego, CA), anti-phospho FAK (Cell Signaling Technologies, Danvers, MA), rabbit anti-ILK (Millipore, Billerica, MA), rabbit anti-EGFR (Cell Signaling Technologies, Danvers, MA), mouse anti- β 1 integrin (BD Transduction, BD Biosciences, San Diego, CA), mouse anti- α 5 integrin (BD Transduction, BD Biosciences, San Diego, CA), mouse anti- α 6 integrin (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-cortactin (Millipore, Billerica, MA), rabbit anti-phospho-VASP Ser 157 (Cell Signaling Technologies, Danvers, MA), rabbit anti-phospho-cofilin Ser3 (Cell Signaling Technologies, Danvers, MA), mouse anti- α Tubulin (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-Rac1 (BD Transduction, BD Biosciences, San Diego, CA), mouse anti-RhoA (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-PINCH1 (GenWay Biotech, San Diego, CA), mouse anti-PINCH1 (Sigma-Aldrich, St. Louis, MO), rabbit anti-PDE4D5 (Thermo Scientific, Rockford, IL) and mouse anti-RACK1 (BD Transduction, BD Biosciences, San Diego, CA). Anti-coronin 1B was a generous gift from Dr. James E. Bear. Anti-profilin, anti-Arp3 and anti- α actinin were a kindly gift from Dr. Michael Schell. The anti-aminoterminal Rsu1 antibody has been described (Dougherty et al., 2008).

Migration Assays

Cells treated with siRNA were seeded in Oris™ migration plates (Platypus Technologies, Madison, WI) at a concentration of 3×10^4 cells per ml. Seventy hours post transfection stoppers were removed vertically from each well with the Oris™ stopper tool (Platypus Technologies, Madison, WI). Media was removed from wells and replaced with 100 μ l of fresh MCF-10A culture media supplemented with 10 ng/ml of EGF. The migration plate was incubated in a humidified chamber (37°C, 5% CO₂) to allow cell migration. At 21 hours post migration initiation the wells were fixed with 3.7% formaldehyde. Quantitation was performed by staining cells with crystal violet and reading absorbance at 570 nm. The number of migrating cells was calculated as the average of 4 wells minus an internal control (stoppers removed right before fixation). Cells were photographed at the beginning and conclusion of the assay.

Migration was also measured by the use of migration plates containing a dissolving gel in each well. Inhibitors or activators were added to MCF-10A cells. Cells were immediately seeded into Oris™ Pro 96 well migration plates (Catalog # PROCMA5, Platypus Technologies, Madison, WI) at a concentration of 8×10^4 per ml. The migration plate was incubated in a humidified chamber (37°C, 5% CO₂) to allow cell migration. At 24 hours post migration initiation the wells were fixed with 3.7% formaldehyde and stained with crystal violet. The diameter of the detection zone was measured using the Q Capture Pro software. The detection zone is the central area of the well where cells have not yet migrated. The number of migrating cells was calculated as the average of 4 wells minus an internal control. Cells were photographed at the beginning and conclusion of the assay.

Adhesion, MTT and Viability Assays

Adhesion was measured as previously described (Dougherty et al., 2005). In brief, MCF-10A cells were transfected with a control, Rsu1 or PINCH1 siRNA. At 72 hours post-transfection, cells were harvested and replated in a 96 well plate at a concentration of 5×10^4 cells per ml. At 10 minute intervals unattached cells were removed and wells were fixed with 3.7% formaldehyde. Quantitation was performed by staining cells with crystal violet and reading absorbance at 570 nm.

Proliferation of MCF-10A cells was determined by an MTT assay (Cell Titer 96 Assay, Promega, Madison, WI). siRNA transfected cells were plated in quadruplicate wells in a 96-well plate. Cells were incubated at 24, 48, 72 and 96 hours post transfection and analyzed as previously described (Cerrito et al., 2004).

Viability of MCF-10A cells was measured by analysing changes in the reduction of alamar blue (AbD Serotec, Raleigh, NC). siRNA transfected cells were plated in quadruplicate wells of a 96-well plate. Ninety-six hours post-transfection, alamar blue was added to each well (10% of total volume) and plates were incubated in a humidified chamber (37°C, 5% CO₂) for 6 hours. Viability was measured by calculating the percentage reduction of alamar blue using absorbance at a wavelength of 570 nm and 600 nm.

Statistical Analysis

The data is displayed as mean +/- SE. Significance was measured by Student's t-test.

Detection of GTP-Rac and GTP-Rho

Rac and Rho GTPase activity assays were performed as previously described (Dougherty et al., 2008). In brief, MCF10A cells were transfected with a control, Rsu1 or PINCH1 siRNA and plated on fibronectin-coated tissue culture dishes. At 96 hours post-transfection cells were stimulated with EGF (50ng/ml) for 15 minutes. The level of GTP-bound Rac1 was determined by a Rac1 effector bead binding assay. The cell lysates were collected and bound to the glutathione beads loaded with the GST-fusion of the Rac1/cdc42 binding domain of Pak. The bound Rac1, “active Rac1”, was detected by western blot with anti-Rac1 monoclonal antibody and compared to the amount of GDP-+GTP-bound Rac1, “total Rac1”, in aliquots of lysates removed prior to bead binding. The amount of active Rac1 was quantified by densitometry.

Immunofluorescence Microscopy

MCF-10A cells were seeded on fibronectin coverslips (BD Transduction, BD Biosciences, San Diego, CA), and assayed for immunofluorescence as described previously (Dougherty et al., 2008). In brief, cells were rinsed in phosphate buffered saline (PBS), and then fixed with cold methanol for 30 min at -20°C or with 4% paraformaldehyde for 15 min at room temperature (RT). Methanol-fixed cells were washed three times in PBS and then blocked in 4% BSA for 1 hour at RT or 4°C overnight. Paraformaldehyde fixed cells were washed three times in PBS and incubated in 0.5% Triton X-100 for 10 min. After permeabilization, cells were washed three times in PBS and blocked in 4% BSA. Primary and secondary antibodies were diluted in 4% BSA. Anti-aminoterminal Rsu1 rabbit polyclonal, mouse anti-Talin (Sigma-Aldrich, St.

Louis, MO), mouse anti-Paxillin (BD Transduction, BD Biosciences, San Diego, CA), mouse anti-Vinculin (Sigma-Aldrich, St. Louis, MO), TRITC-Phalloidin (Sigma-Aldrich, St. Louis, MO), rabbit anti-Myc Tag (Cell Signaling Technologies, Danvers, MA), mouse anti-Caveolin (BD Transduction, BD Biosciences, San Diego, CA), mouse anti- β 1 integrin, rabbit anti-PDE4D5 (Thermo Scientific, Rockford, IL) and mouse anti-RACK1 (BD Transduction, BD Biosciences, San Diego, CA) antibodies were used for immunofluorescence analysis. Anti-EEA1 and Anti-Transferrin receptor antibodies were a generous gift from Dr. Gudrun Ihrke. Anti-Coronin 1B was a generous gift from Dr. James E. Bear. Anti-profilin, anti-Arp3 and anti- α Actinin were a kind gift from Dr. Michael Schell. Alexa-Fluor conjugated antibodies were used as secondary antibodies (Molecular Probes, Invitrogen, Eugene, OR). Coverslips were mounted on slides with ProLong Gold antifade reagent (Molecular Probes, Invitrogen, Eugene, OR). Images were taken with an Olympus IX71 and Zeiss 710 Confocal Laser Scanning Microscope. Images were acquired and analyzed with QCapture Pro 6.0 and Zeiss LSM software. For each slide, images were obtained from random fields. Representative images were selected that displayed the phenotype consistent with the majority (over 90%) of the random fields.

Dextran uptake assay

Changes in endocytosis were measured by using the Dextran uptake assay. A FITC-labeled Dextran was a generous gift from Dr. Gudrun Ihrke. Transiently transfected cells were washed with pre-warmed PBS and then incubated with FITC-Dextran at 37°C for 1 hour. This time point allows for the detection of changes in endocytosis of late

endosomes. Following the one-hour incubation, cells were fixed and processed for immunofluorescence as described above.

Lentivirus construction and viral infection

An Rsu1 mutant that does not bind to PINCH1 (N91D) was created by site directed mutagenesis. Co-immunoprecipitation and yeast two hybrid assays were performed to confirm that the mutant does not bind to PINCH1 as described previously (Dougherty et al., 2005). Rsu1 wt and Rsu1-N91D were introduced into lentiviral vectors, pSMPU.

Lentivirus was produced by transfection of control (empty vector), Rsu1 (wt) or N91D into 293T cells along with a VSV-G and polymerase ($\Delta 8.2$) plasmid. Virus was harvested 72 hours post-transfection and filtered through a 0.45 μ M filter (Millipore, Billerica, MA). Polybrene was added to the virus prior to infection.

MCF-10A cells were transfected with a control or Rsu1 (UTR) siRNA and seeded into a migration plate and fibronectin coverslips for migration and immunofluorescence studies, respectively. Twenty-four hours post-transfection, MCF-10A cells were infected with a lentivirus encoding a control (empty vector), Rsu1 (wt) or N91D for a period of 8 hours. Media was then removed and replaced with MCF-10A fresh medium. At 70 hours post-infection, the migration assay was initiated. At 96 hours, the migration assay was stopped, fibronectin coverslips were processed for immunofluorescence and lysates were collected for Western blot analysis.

Inhibitor and Activator Treatments

MCF-10A cells were treated with inhibitors for 24 hours at the following concentrations: EGF-R (20 μ M AG-1478), PI3K (10 μ M LY29402), Mek (20 μ M U0126), Rap (25 μ M GGTi), PKC (10 nM Bim), Rac (25 μ M NSC-23766), JNKII (100 nM SP600125) and PKA (10 μ M H89). Alternatively, cells were treated with activators for 24 hours at the following concentrations: PKA-EPAC activator (100 μ M 8-Bromo cAMP), Adenyl Cyclase activator (10 μ M Forskolin) and EPAC activator (50 μ M 8-CPT-2'Me-cAMP).

cAMP Assay

Levels of cAMP were measured by a cAMP ELISA kit (Enzo Life Sciences). Control and Rsu1 depleted cells were plated in a 96 well ELISA plate. The plate was processed according to the manufacturer's protocol. cAMP levels were measured by reading absorbance at 405 nm.

Co-immunoprecipitation

Immunoprecipitations were performed as previously described (Dougherty et al., 2008). Immunoprecipitates were collected with a N-terminal Rsu1 polyclonal antibody (Dougherty et al., 2008).

Chapter 3: Results

Rsu1 or PINCH1 depletion decreased MCF-10A cell adhesion and migration.

Rsu1 or PINCH1 siRNA treatment resulted in a decrease in their respective protein levels by over 80% as confirmed by Western blot analysis. In addition, depletion of Rsu1 also reduced PINCH1 by 70% (**Figure 3A**). Consistent with previous studies (Dougherty et al., 2005; Dougherty et al., 2008), reduction of Rsu1 or PINCH1 proteins resulted in a decrease in adhesion to tissue culture plastic in MCF-10A cells (**Figure 3B**). Cells depleted of Rsu1 or PINCH1 exhibited a reduction in EGF-stimulated cell migration as measured in quantifiable assay plates (**Figure 3C**). This assay examined cell migration for a 24 hour period initiated at 72 hours post siRNA transfection. Rsu1 depletion did not affect cell proliferation, while reduction in PINCH1 decreased MCF-10A proliferation (**Figure 3D**). There was no difference in the viability of Rsu1- or PINCH1-depleted cells based on alamar blue uptake (**Figure 4**).

Rsu1 depletion did not alter PINCH1 localization, implying that binding to Rsu1 is not required for PINCH1 localization (**Figure 5A**). In contrast, PINCH1 depletion resulted in a disruption in the localization of Rsu1 to FAs, suggesting that FA-associated PINCH1 is necessary for Rsu1 migration to these sites (**Figure 5A**).

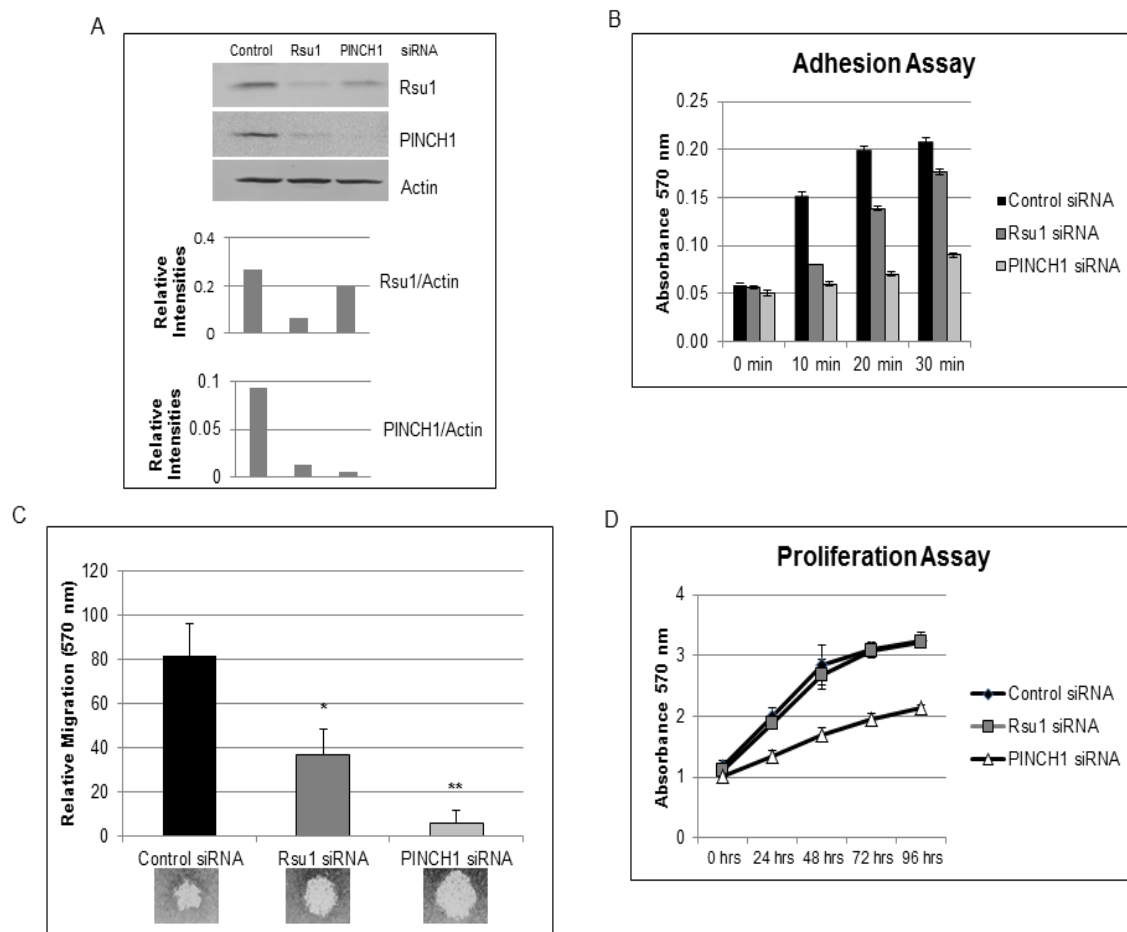


Figure 3. Rsu1 and PINCH1 are required in adhesion and migration. MCF-10A cells were transfected with a Control, Rsu1 or PINCH1 siRNA. (A) Verification of knockdown was confirmed by Western blot analysis. (B) 96 hours post-transfection, cells were trypsinized and re-plated in a 96 well plate. Cells were fixed and stained with crystal violet at 10, 20 and 30 minutes. Adhesion was determined by reading absorbance at 570 nM. All time points were done in triplicate. (C) siRNA-treated cells were seeded in migration plates containing an insert that was removed 72 hours-post transfection. Migration plates were incubated at 37°C for an additional 24 hours to allow documentation and quantification of cell migration. Quantification was performed by staining cells with crystal violet and reading absorbance at 570 nM with a template for the field of migration. * $P < 0.05$, ** $P < 0.01$. The results are displayed as the mean of 4 independent wells \pm SE. (D) siRNA treated cells were seeded in a 96-well microtiter plate. Cell proliferation was measured at 24, 48, 72 and 96 hours by an MTT assay.

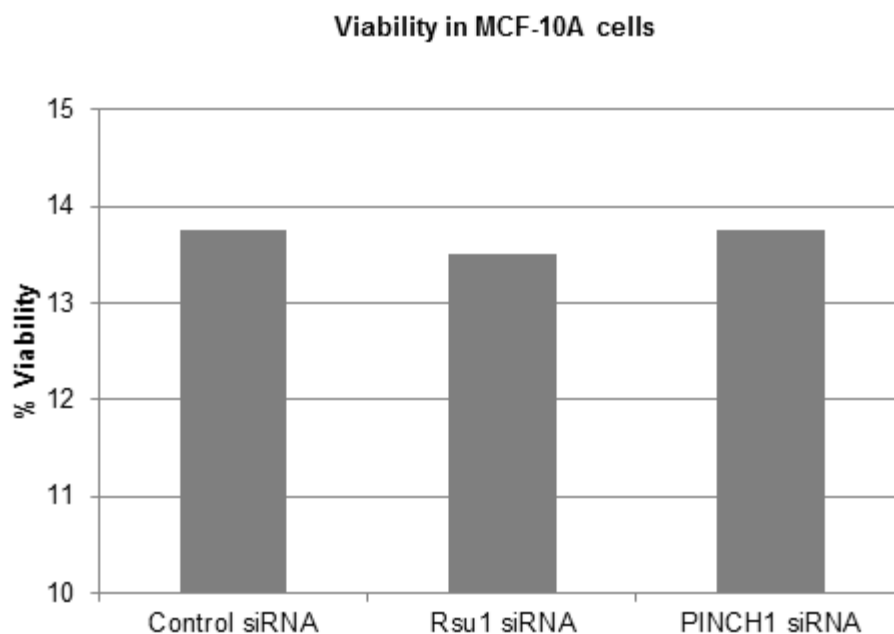


Figure 4. Rsu1 or PINCH1 depletion does not affect the viability of MCF-10A cells. MCF-10A cells were transfected with a Control, Rsu1 or PINCH1 siRNA and plated in quadruplicate wells in a 96-well plate. At 96 hours post-transfection, alamar blue was added to each well (10% of total volume). Plates were incubated at 37 °C for 6 hours. Absorbance was measured at a wavelength of 570nm and 600 nm. % of viability was calculated according to the manufacturer's protocol.

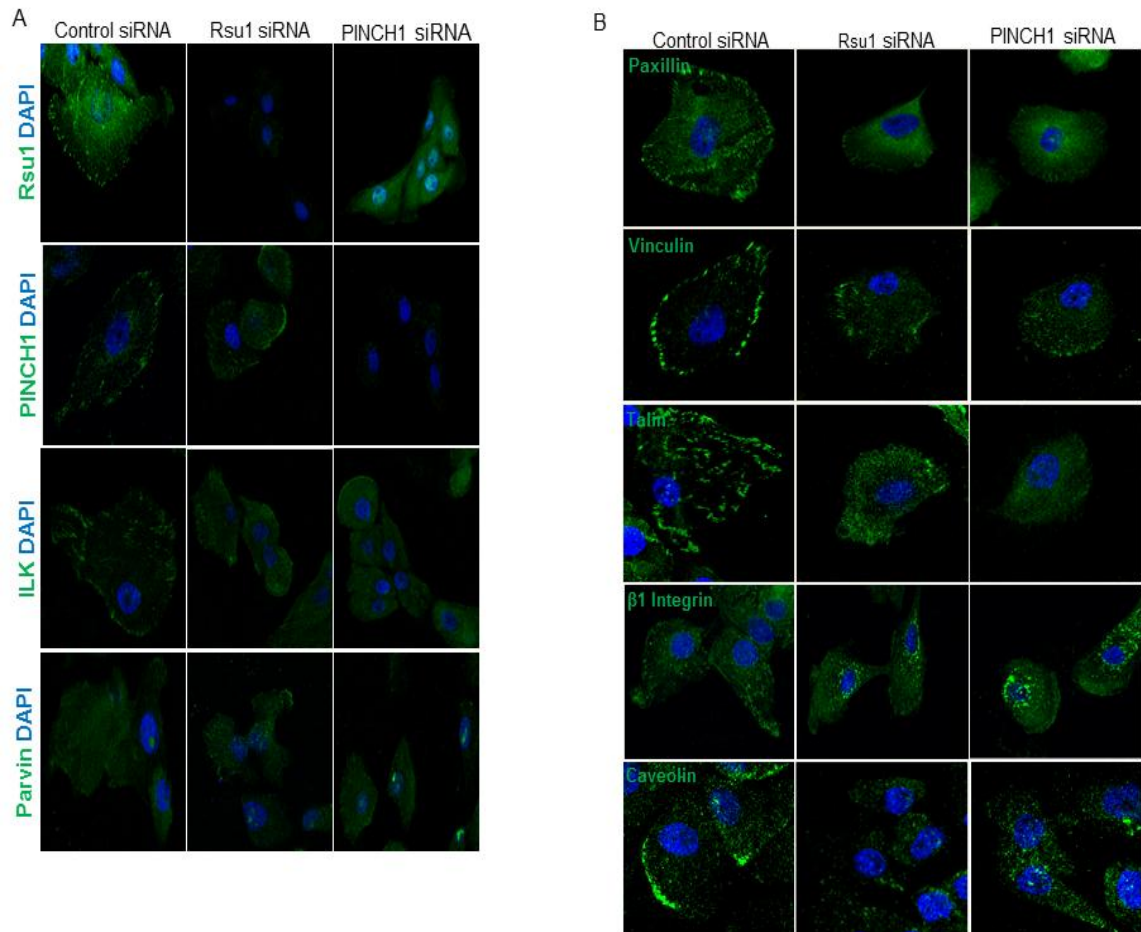


Figure 5. The distribution of common focal adhesion proteins is dependent on an intact RIPP complex. MCF-10A cells were transfected with a Control, Rsu1 or PINCH1 siRNA and plated on fibronectin coverslips. Cells were fixed at 96 hours post-transfection and assayed by immunofluorescence using anti-Rsu1, anti-PINCH1, anti-ILK, anti-Parvin, anti-Paxillin, anti-Vinculin, anti-Talin, anti- $\beta 1$ integrin and anti-caveolin (A-B). Nuclei were counterstained with DAPI.

An intact RIPP complex is required for the formation of focal adhesions

Previous studies conducted in our laboratory demonstrated that Rsu1 and the IPP complex localized to focal adhesions (FA) sites in cells (Dougherty et al., 2008). Because loss of Rsu1 or PINCH1 resulted in a decrease in cell adhesion and migration (**Figure 3**), we examined the impact of Rsu1 or PINCH1 depletion on the expression of focal adhesions and focal adhesion proteins. Depletion of Rsu1 or PINCH1 in MCF-10A cells altered the distribution and localization of the FA proteins ILK, vinculin, paxillin and talin (**Figure 5**). Control cells displayed a peripheral and highly organized staining of focal adhesion proteins while the Rsu1- or PINCH1-depleted cells exhibited retraction of the cell body and disorganized focal adhesion protein staining throughout the cytoplasm.

We next examined the role of Rsu1 and PINCH1 in focal adhesion formation. Rsu1- or PINCH1-depleted cells were immunostained for vinculin and phalloidin to determine formation of focal contacts and adhesion complexes at 30, 60 and 120 min after replating cells on fibronectin (**Figure 6**). Cell spreading was augmented in Rsu1- or PINCH1-depleted cells. In addition, the actin cytoskeleton did not associate with the FAs like the control cells exhibited. These results indicate that Rsu1 and PINCH1 are critical for the formation of focal adhesions and the proper localization of focal adhesion proteins.

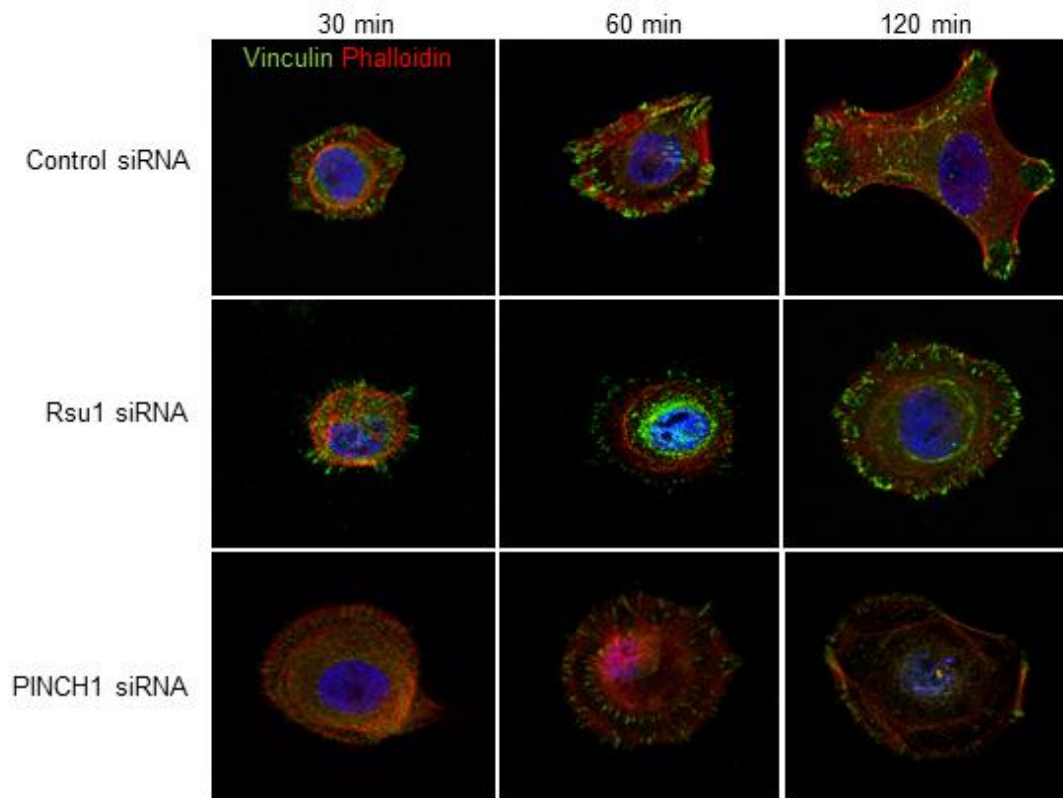


Figure 6. Rsu1 and PINCH1 are required for the formation of FAs. MCF-10A cells were transfected with a Control, Rsu1 or PINCH1 siRNA. At 96 hours post-transfection, cells were trypsinized and re-plated onto fibronectin coverslips. Cells were fixed at 30, 60 and 120 minutes and assayed by immunofluorescence using TRITC phalloidin and anti-vinculin antibodies. Nuclei were counterstained with DAPI.

Since the IPP complex has been shown to be an important regulator of integrin signaling we examined the effect of Rsu1 or PINCH1 depletion on the distribution and localization of $\beta 1$ integrin (Cabodi et al., 2010; Legate et al., 2006) (**Figure 5B**). In addition, caveolin was analyzed because of its pivotal role in receptor-independent endocytosis, integrin recycling and its association with the RIPP complex (Parton et al., 2006; Parton and Simons, 2007; Wickstrom et al., 2010a). MCF-10A cells depleted of Rsu1 or PINCH1 adhered poorly to substrate and exhibited disorganized caveolae (**Figure 5B**) and the loss of visible $\beta 1$ integrin complexes (**Figure 5B**). We next investigated the effect of Rsu1 or PINCH1 depletion on the recycling machinery by assessing changes in endosomal markers, including an early endosome marker (EEA1) and a recycling endosome marker (transferrin receptor). There was limited change in the localization of endosomal markers between control and Rsu1-depleted cells (**Figure 7A**). In addition, control and Rsu1-depleted cells were treated with dextran to establish any changes in the endocytic and recycling pathway. Although the uptake of dextran was slightly different in Rsu1 and PINCH1-depleted cells, the change was not sufficient to explain the large scale loss of focal adhesions (**Figure 7B**). Co-staining of $\beta 1$ integrin with a FITC-labeled dextran revealed that the accumulation of $\beta 1$ integrin seen in the Rsu1 and PINCH1-depleted cells colocalized with late endosomes (**Figure 7B**). Together these results suggest that Rsu1 or PINCH1 depletion affects the localization of integrins, which is only partially linked to a defect in the recycling process.

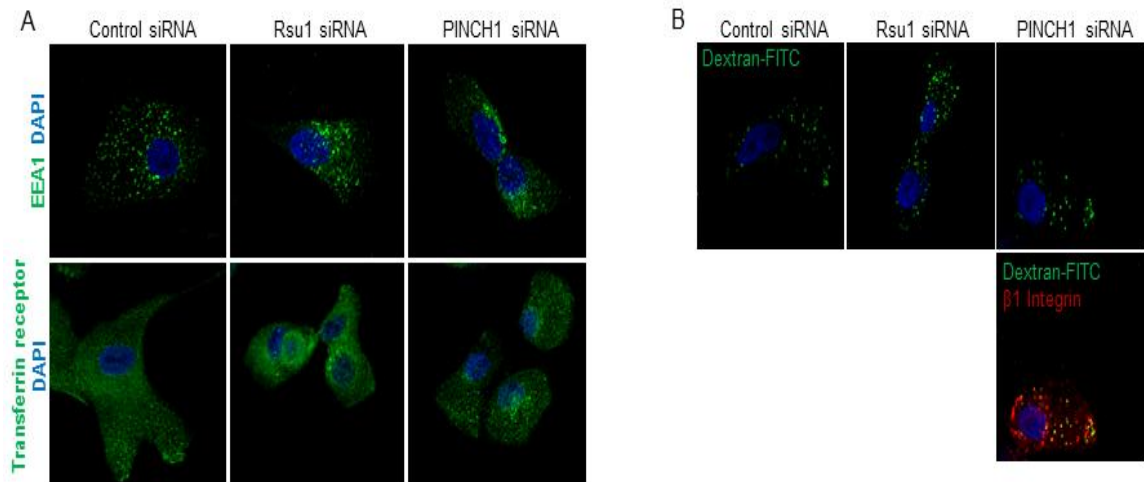


Figure 7. Rsu1 and PINCH1 have a role in endocytic transport. MCF-10A cells were transfected with Control, Rsu1 or PINCH1 siRNA and plated on fibronectin coverslips. (A) Cells were fixed at 96 hours and assayed by immunofluorescence using EEA1 and transferrin receptor antibodies. Nuclei were counterstained with DAPI. (B) At 96 hours post-transfection, transiently transfected cells were washed with pre-warmed PBS and then incubated with FITC-Dextran at 37°C for 1 hour. One hour post-treatment cells were fixed and processed for immunofluorescence. Co-staining with Dextran was done using an anti- $\beta 1$ integrin antibody.

Rsu1 or PINCH1 depletion did not affect the expression of the majority of the focal adhesion proteins and integrins

The expression of the members of the RIPP complex (Rsu1, PINCH1, Parvin), focal adhesion proteins (Paxillin, Vinculin, Talin, and FAK), integrins ($\alpha 6$, $\alpha 5$, $\beta 1$), caveolin and EGF-R was determined by Western blot analysis. Rsu1 or PINCH1 depletion did not affect the expression of the proteins mentioned above with the exception of $\beta 1$ integrin, p-FAK, FAK and EGF-R (**Figure 8**). $\beta 1$ integrin and FAK expression was elevated upon Rsu1 and PINCH1 knockdown, while EGF-R, and p-FAK levels were dramatically reduced (**Figure 8**). This elevation could be a compensatory response of Rsu1- and PINCH1-depleted cells for the inhibition of adhesion. Some of the $\beta 1$ integrin binding partners expressed in MCF-10A cells include $\alpha 5$, $\alpha 6$ and αv (Barczyk et al., 2010; Haenssen et al., 2010; Plopper et al., 1998) , and their expression was not altered.

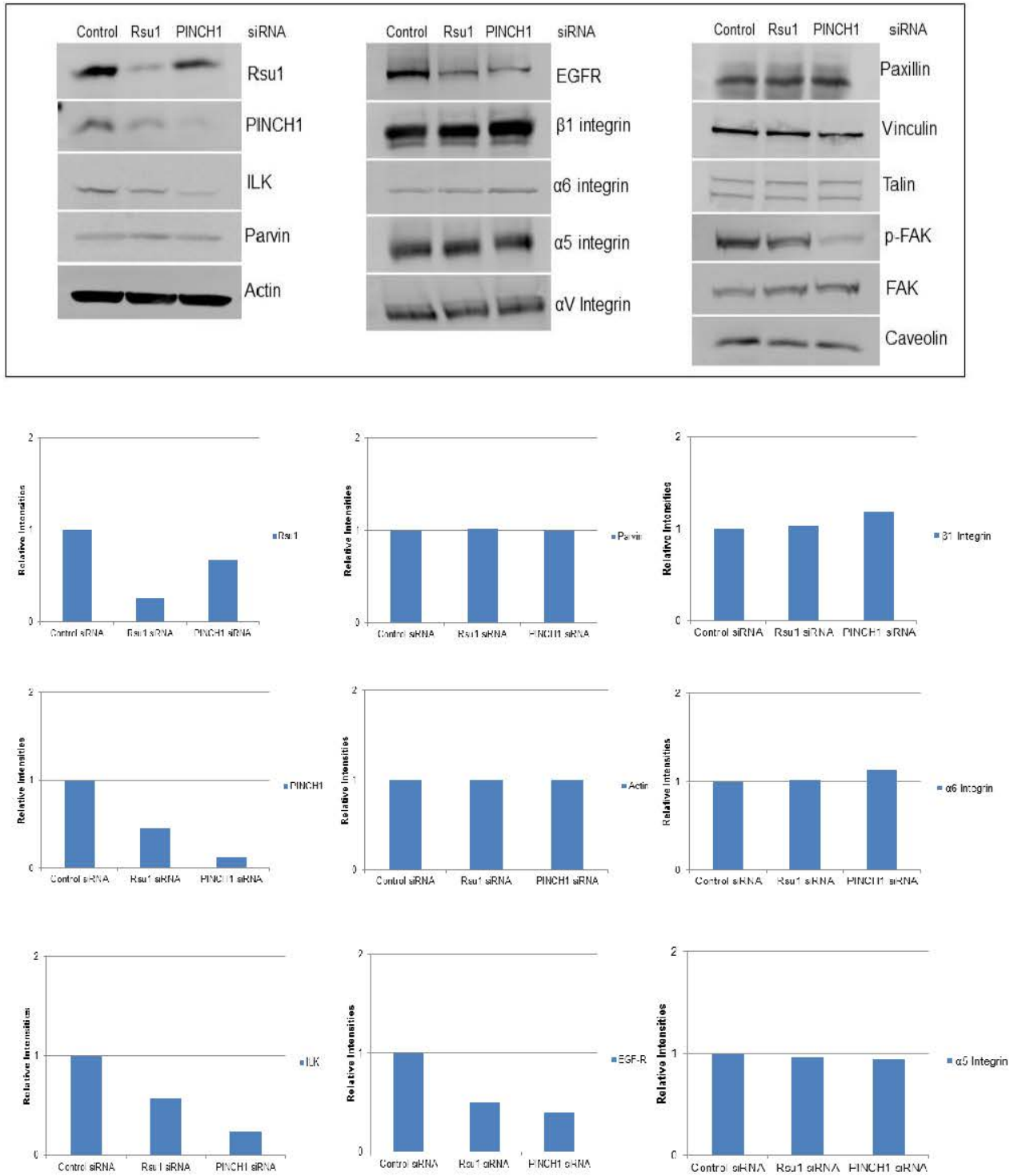
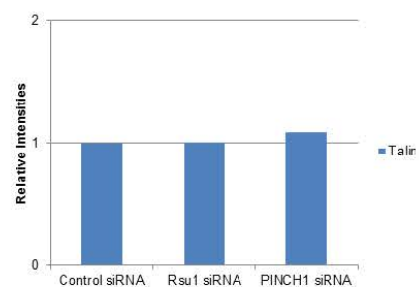
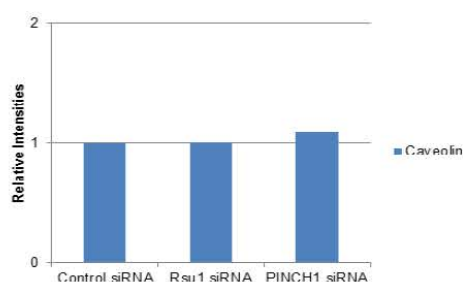
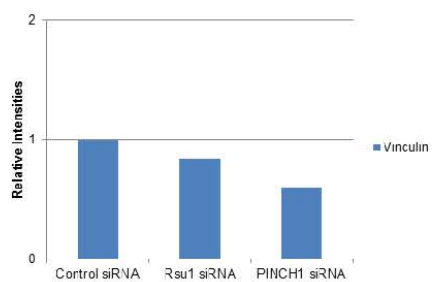
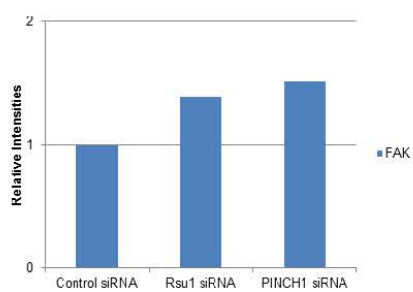
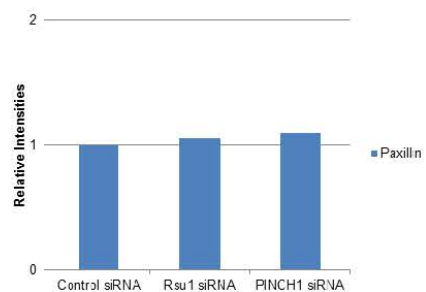
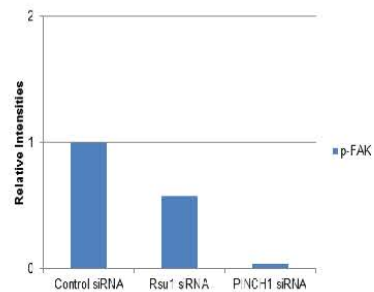
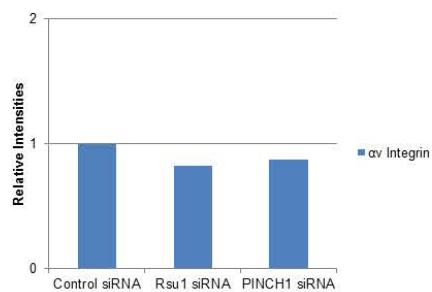


Figure 8. Expression of most FA proteins and integrins is not affected by Rsu1 or PINCH1 depletion. Lysates (100 μ g in RIPA buffer) of MCF-10A cells transfected with a Control, Rsu1 or PINCH1 siRNA were harvested 96 hours post transfection and examined for the expression of Rsu1, PINCH1, ILK, Parvin, EGF-R, Caveolin, Paxillin, Vinculin, Talin, Integrins ($\alpha 5$, $\alpha 6$, αv , $\beta 1$), p-FAK and FAK by Western blotting. Actin was used as loading control. Western blot was quantified by densitometry.



Disorganized focal adhesion staining in Rsu1 and PINCH1-depleted cells correlated with loss of stress fibers and elevated expression of actin binding proteins but not with changes in the activation of small G proteins.

Phalloidin labeling was used to assess changes in actin polymerization in response to loss of Rsu1 or PINCH1. Rsu1 or PINCH1 depletion resulted in loss of stress fibers and disorganized actin cytoskeleton staining (**Figure 9A**). A reorganization of the actin cytoskeleton correlated with loss of FAs in Rsu1- and PINCH1-depleted cells. We next investigated the expression and localization of actin binding proteins in the hope of finding a difference that could explain the changes seen in the actin cytoarchitecture of Rsu1 and PINCH1-depleted cells. A deregulation in the localization of the actin binding proteins was observed upon Rsu1 or PINCH1 knockdown (**Figure 9B**). As shown in **Figure 9C**, depletion of Rsu1 or PINCH1 resulted in a mild increase in the expression of actin associated protein Cortactin, Arp3 and Coronin 1B and an increase in phosphorylation of VASP and Cofilin on sites that control their actin polymerization/capping activity. Rsu1 or PINCH1- depleted cells also expressed a reduction in the expression of the crosslinker actin binding protein α -actinin. This protein is involved in the formation of actin stress fibers (Legate et al., 2006). The constitutive phosphorylation and the change in the localization and distribution of actin regulatory proteins that occurred in Rsu1- or PINCH1-depleted cells suggest a role of Rsu1-PINCH1 binding in the regulation of actin polymerization.

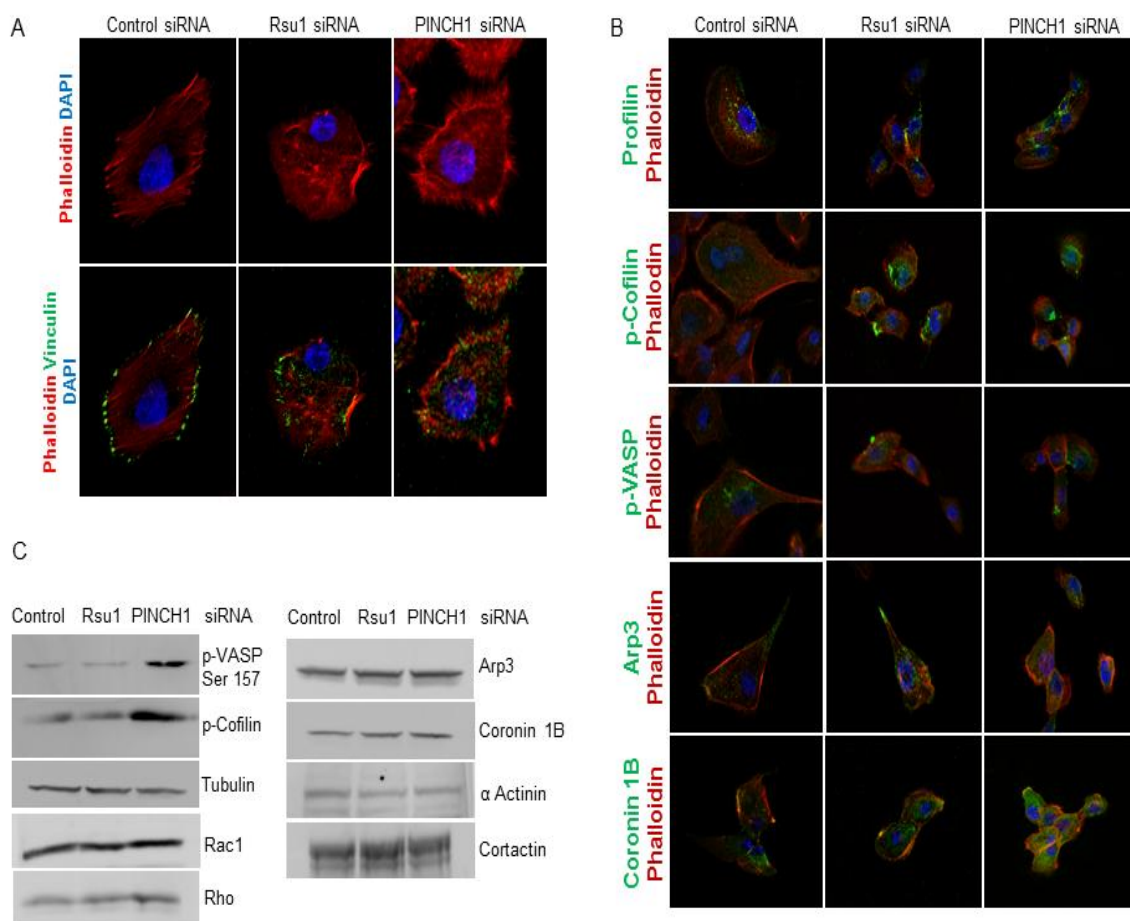
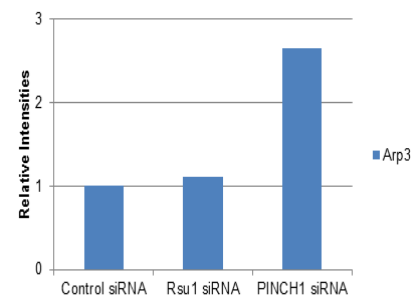
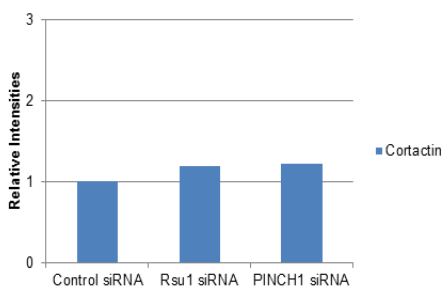
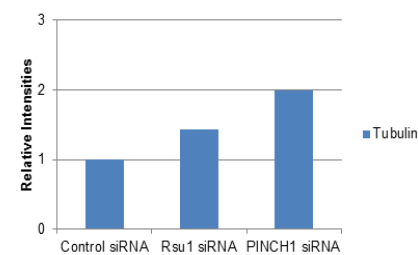
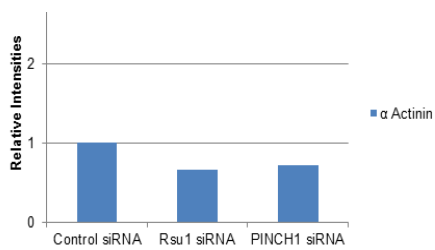
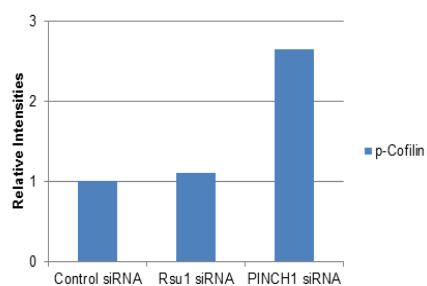
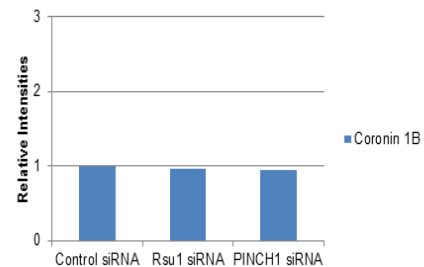
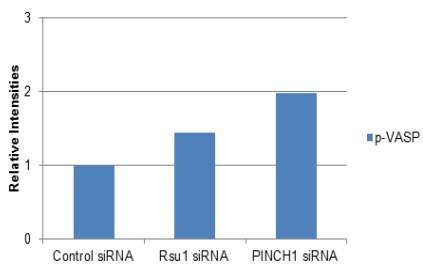
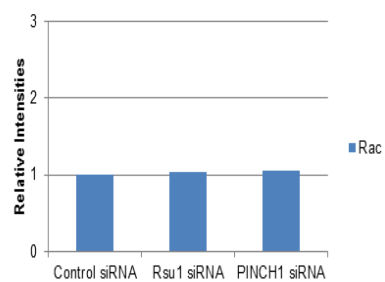
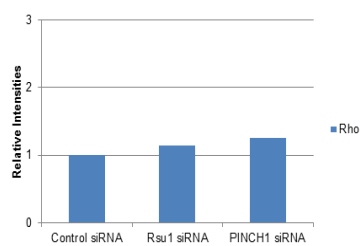


Figure 9. Disorganized focal adhesion staining correlates with reorganization of the actin cytoskeleton in Rsu1 and PINCH1 depleted cells. MCF-10A cells were transfected with a Control, Rsu1 or PINCH1 siRNA and plated on fibronectin coverslips. (A) Cells were fixed at 96 hours post-transfection and assayed by immunofluorescence using TRITC phalloidin and anti-vinculin antibody. (B) (C) Rsu1 and PINCH1 depleted cells were fixed at 96 hours post-transfection and assayed by immunofluorescence using TRITC phalloidin, Arp3, Coronin 1B, Profilin, p-Cofilin, and p-VASP. Nuclei were counterstained with DAPI. (C) Lysates were harvested 96 hours post-transfection and examined for expression of p-VASP, p-Cofilin, Coronin 1B, Cortactin, Arp3, Rac1, Rho and α -Actinin. Tubulin was used as loading control. Western blot was quantified by densitometry.



The Rho family of GTPases is actively involved in the process of cell migration by regulating actin reorganization (Iden and Collard, 2008; Yamazaki et al., 2005). Since Rsu1 and members of the IPP complex affect Rac1 and RhoA activation, we determined the level of Rac1-GTP and RhoA-GTP by pulldown activity assays (Dougherty et al., 2008; Vasaturo et al., 2000). Activation of G proteins by growth factor is not affected by Rsu1 or PINCH1 siRNA mediated depletion (**Figure 10**). Together these results demonstrated a critical role for Rsu1 and PINCH1 in actin polymerization. However, the Rsu1- or PINCH1-depleted cells retained the ability to activate Rac1 and Rho in response to EGF stimulation, which is important since migration is an EGF-driven process.

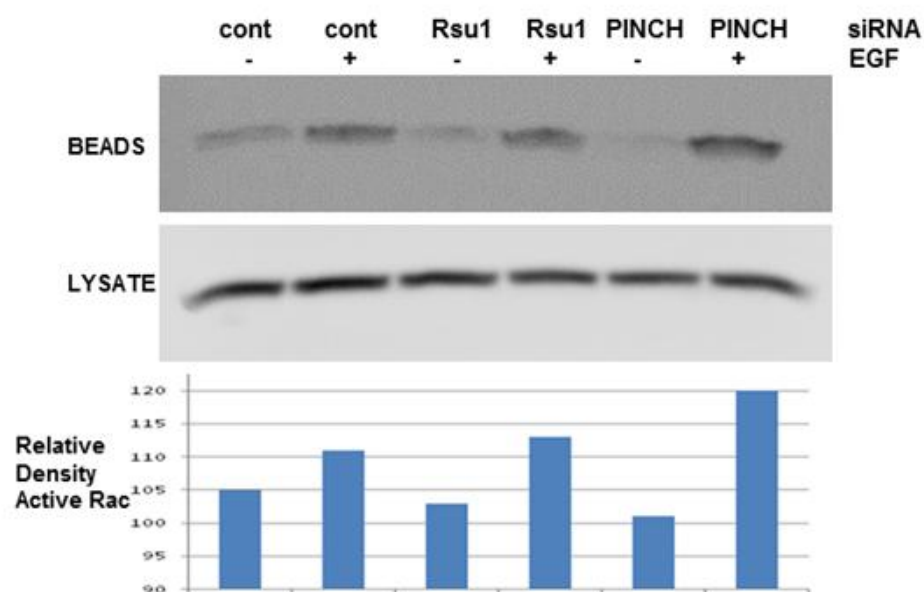


Figure 10. The inhibitory effect of Rsu1 or PINCH1 depletion on Rac1 activation was mitigated by EGF. MCF10A cells were transfected with the indicated siRNA and plated on fibronectin-coated tissue culture dishes. At 96 hours post-transfection cells were harvested from wells with or without EGF stimulation (50ng/ml, 15 minutes). The level of GTP-bound Rac1 was determined by binding to the GST-Rac1-cdc42 binding domain of Pak1 (GST-PBD). The amount of active Rac1 was quantified by densitometry.

An Rsu1 mutant that does not bind to PINCH1 restored adhesion and migration in Rsu1 knockdown cells

Rsu1 binds to PINCH1 to link it to the IPP complex, and reductions in Rsu1 result in a corresponding decrease in PINCH1. Thus, investigating the ability of Rsu1 to regulate adhesion and migration independently from its binding to PINCH1 and its association with the IPP complex is an important subsequent study. Consequently, an Rsu1 mutant that does not bind to PINCH1 was created in order to determine whether or not the mutant can rescue adhesion and migration to the same degree as the wt Rsu1. As shown in **Figure 11A**, Cos1 cells transfected with vectors containing wt Rsu1 or a mutant Rsu1 (N91D) were not able to bind to PINCH1. In addition, yeast two hybrid data confirmed that the Rsu1 N91D mutant does not bind to PINCH1 (Data not shown).

To establish if the Rsu1-N91D mutant can rescue migration in Rsu1-depleted cells, MCF-10A cells depleted of Rsu1 were infected with a lentivirus expressing Rsu1 wt or Rsu1-N91D. Infection with a lentivirus expressing either Rsu1 wt or Rsu1-N91D restored migration in Rsu1 depleted cells (**Figure 11B**). As shown in **Figure 11C**, cells infected with the lentivirus expressed a band that is ~36kDa (viral product) and the endogenous Rsu1 which encodes a 33kDa product. The increase in migration seen in Figure 5B was accompanied by restoration of FAs, stress fibers and cell spreading in Rsu1 depleted cells (**Figure 12**). Together these results suggest that there is another protein(s) that is interacting directly or indirectly with Rsu1 to compensate for the lack of binding with the PINCH1 complex.

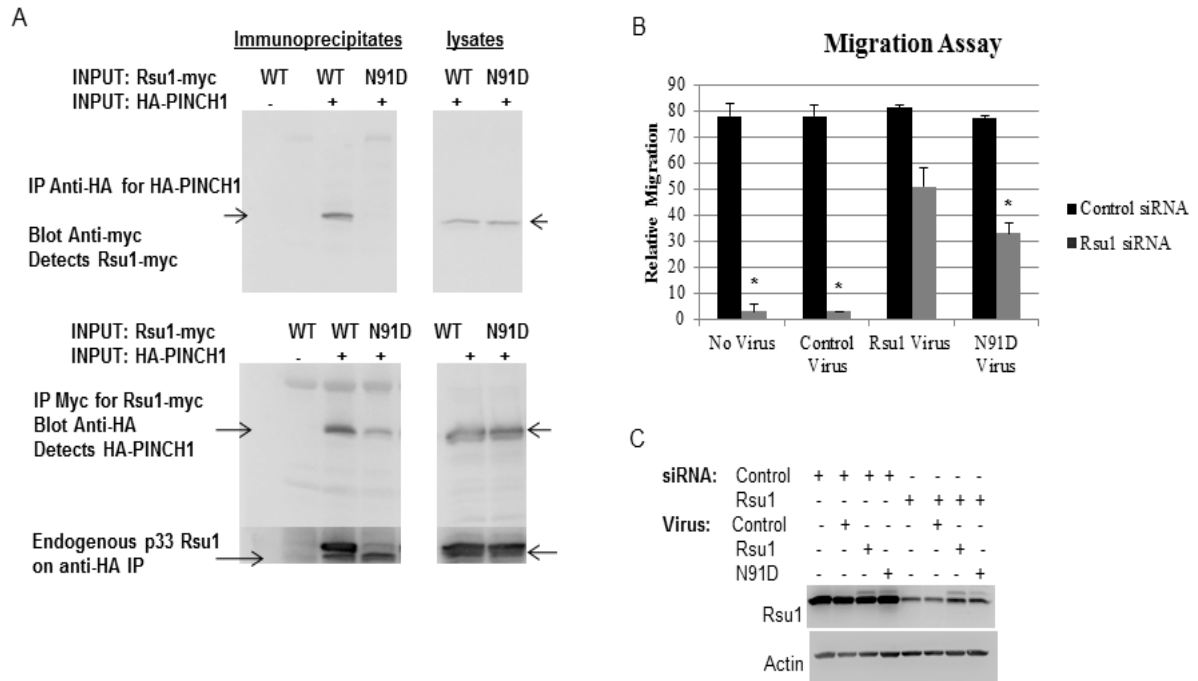


Figure 11. An Rsu1 mutant that does not bind to PINCH1 restored adhesion and migration in Rsu1 depleted cells. (A) 293T cells were transfected with piRES vectors encoding an empty vector, Rsu1 (WT), or an Rsu1 mutant that does not bind to PINCH1 (N91D). At 96 hours, lysates were immunoprecipitated with anti-HA for HA-PINCH1 and anti-Myc for Myc-Rsu1. Co-immunoprecipitation of Rsu1 and the N91D mutant with PINCH1 was analyzed by Western blotting. (B) MCF-10A cells were transfected with a Control or Rsu1 siRNA and seeded into a migration plate, fibronectin coverslips and tissue culture plates for migration, immunofluorescence and Western blot studies, respectively. Twenty-four hours post transfection, MCF-10A cells were infected with a lentivirus encoding a Control (empty vector), Rsu1 (WT) or N91D for 8 hours. Then, media was removed and replaced with MCF-10A fresh medium. (B) The migration assay was initiated at seventy hours post transfection and stopped at 96 hours. Quantification of cell migration was performed by staining cells with crystal violet and reading absorbance at 570 nM. Graphic representations of results from 5 stained wells (Mean+SE). * $P < 0.006$. (C) Rsu1 depletion and virus infection was verified by Western blotting.

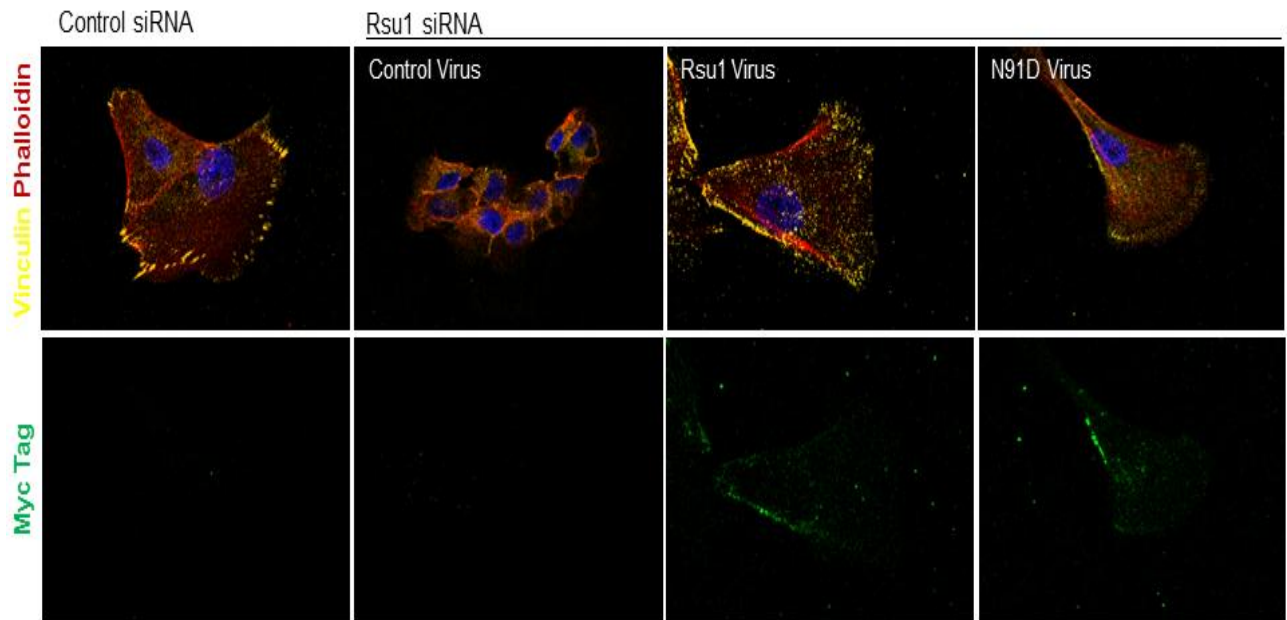


Figure 12. An Rsu1 mutant that does not bind to PINCH1 restored adhesion sites in Rsu1 depleted cells. MCF-10A cells were transfected with a Control or Rsu1 siRNA and seeded onto fibronectin coverslips. Twenty-four hours post transfection, MCF-10A cells were infected with a lentivirus encoding a Control (empty vector), Rsu1 (wt) or N91D for 8 hours. Cells were fixed at 96 hours post-transfection and assayed by immunofluorescence using TRITC-phalloidin, anti-myc tag and anti-vinculin antibodies. Nuclei were counterstained with DAPI. 96% of cells infected with the wt Rsu1 virus and 83% of cells infected with the N91D virus displayed a spread phenotype as well as well-formed FAs. 80% of cells were depleted for Rsu1.

Inhibition of PKA signaling increased migration in MCF-10A cells

An inhibitor approach was used to identify migration effects in MCF-10A cells. We selected inhibitors that target signaling pathways known to be involved in cell migration. Cells treated with inhibitors that target Mek, PI3 Kinase, Rap1, PKC and EGF-R exhibit a decrease in migration while inhibition of Rac and JNK did not have an effect (**Figure 13**). A dramatic increase in migration was observed upon treatment with a PKA inhibitor, H89 (**Figure 13**).

Focal adhesion distribution, localization and actin cytoskeleton cytoarchitecture were examined in cells treated with inhibitors and cells depleted of Rsu1 or PINCH1 with the purpose of comparing the effects of the knockdown versus the inhibitor treatment. Cells treated with inhibitors of the PI3 kinase, PKC or Rap1 displayed larger and more prominent FAs which were accompanied by well-formed stress fibers (**Figure 14**). Conversely, treatment with a PKA inhibitor (H89) resulted in loss of stress fibers and smaller FAs (**Figure 14**). H89 inhibits the kinase activity of PKA by blocking adenosine triphosphate (ATP) site on the PKA catalytic subunit. Together these data showed a function for PKA signaling in the migration of MCF-10A cells.

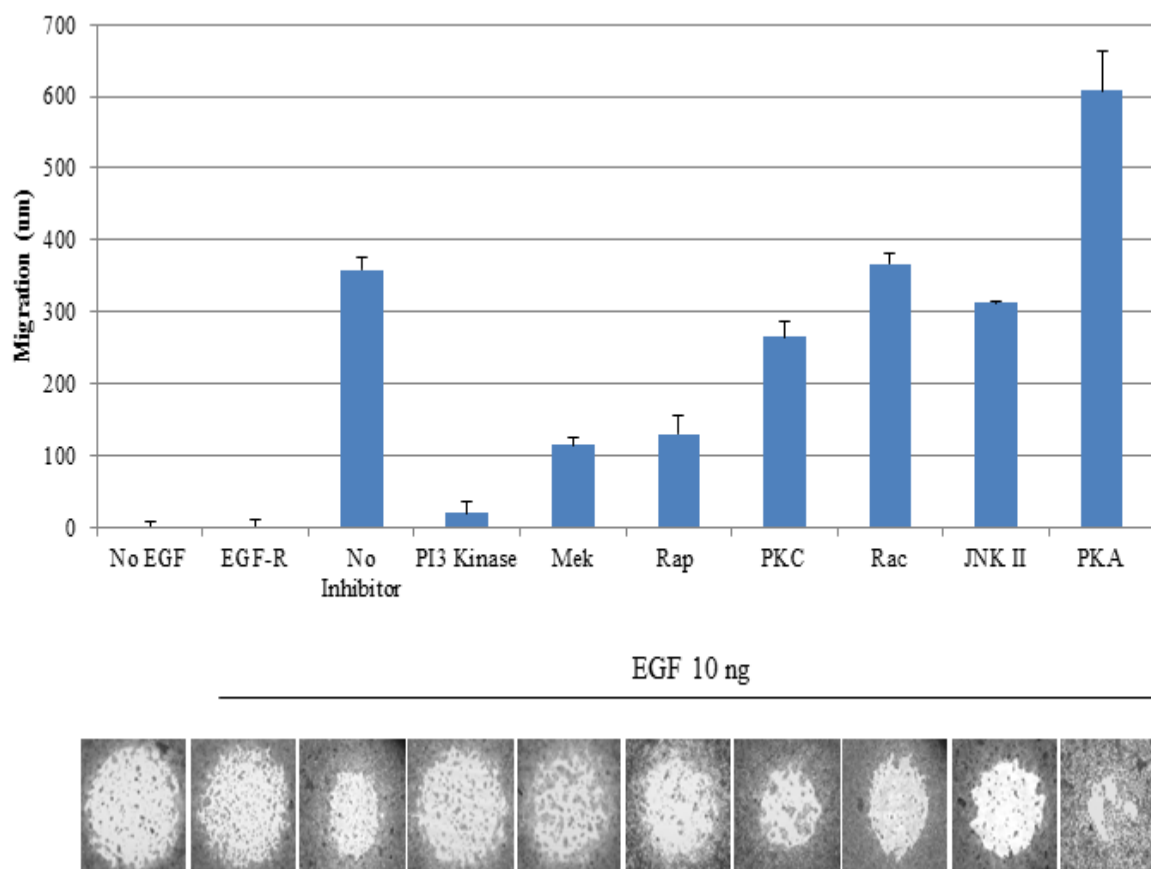


Figure 13. Migration of MCF-10A cells upon inhibitor treatment.

MCF-10A cells were treated with inhibitors and replated in a migration plate.

The plate was incubated in a humidified chamber (37°C, 5% CO₂) for 24 hours to allow cell migration. Cells were then fixed and stained with crystal violet.

Area of migration was calculated using the Q Capture Pro software.

Inhibitors: EGFR (20 μ M AG-1478), PI3K (10 μ M LY29402), Mek (20 μ M U0126), Rap (25 μ M GGTi), PKC (10 nM Bim), Rac (25 μ M NSC-23766), JNKII (100 nM SP600125) and PKA (10 μ M H89).

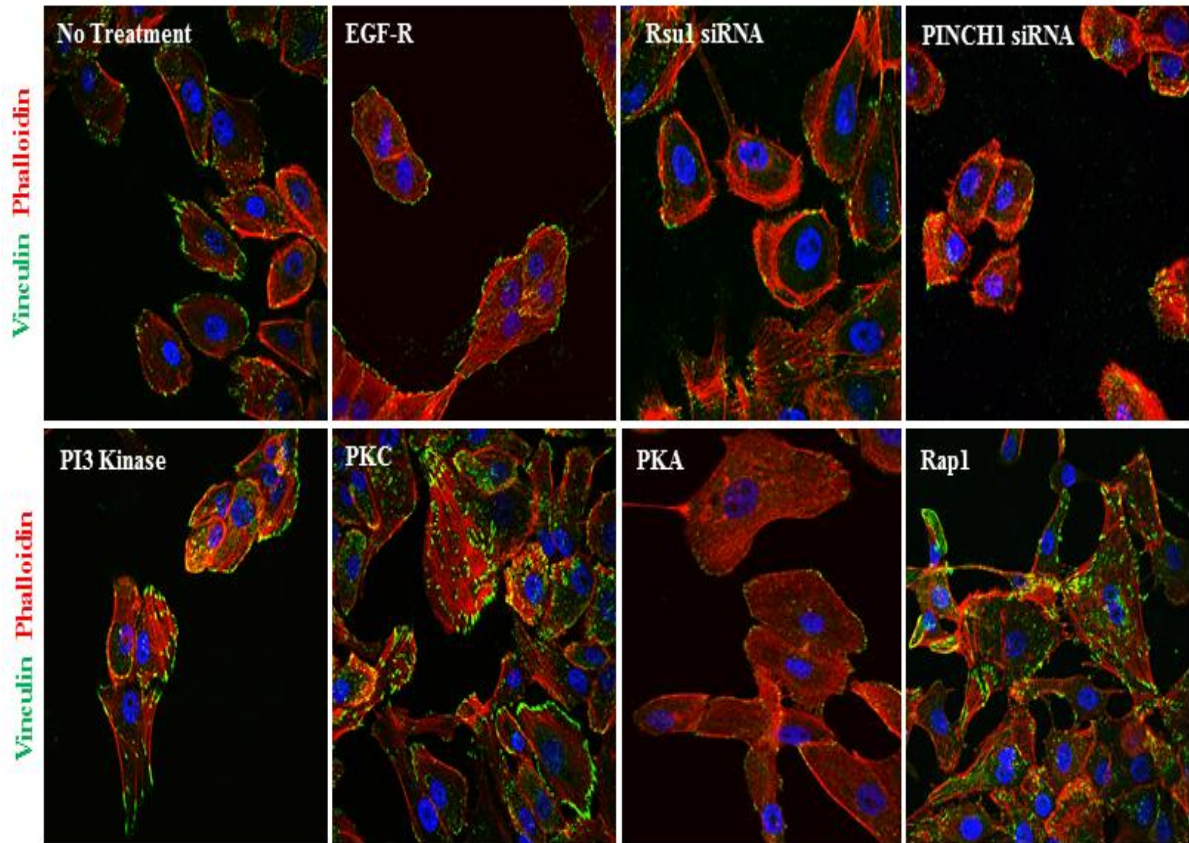


Figure 14. Treatment with inhibitors resulted in a change in FAs and the actin cytoskeleton. MCF-10A cells were plated onto fibronectin coverslips and treated with EGF-R, PI3 kinase, PKC, PKA and Rap1 inhibitors for a period of 24 hours. At 24 hours, cells were fixed and assayed by immunofluorescence using TRITC-phalloidin and vinculin antibodies. Rsu1 and PINCH1 depleted cells were also processed for immunofluorescence 96 hours post-transfection. Nuclei were counterstained with DAPI.

Rsu1 is required to maintain cAMP regulation in MCF-10A cells

Cells undergoing detachment display elevated levels of cAMP (Norambuena and Schwartz, 2011). Since Rsu1 siRNA mediated depletion resulted in an increase in cell detachment, levels of cAMP were measured in Rsu1-depleted cells. Levels of cAMP were determined by the use of a cAMP ELISA kit (Enzo Life Sciences). Rsu1-depleted cells expressed elevated levels of cAMP compared to controls suggesting a role for Rsu1 in cAMP mediated signaling (**Figure 15A**).

Inhibition of PKA and EPAC activation restored migration in Rsu1 depleted cells

Since cAMP signaling is involved in MCF-10A migration, inhibitors or activators of this pathway were used to examine migration effects in Rsu1 depleted cells. MCF-10A cells were transfected with a Control and Rsu1 siRNA. 72 hours post-transfection cells were treated with various inhibitors or activators of cAMP signaling. Consistent with previous data, Rsu1 depletion blocked cell migration in MCF-10A cells (**Figure 15B**). Interestingly, treatment with a PKA inhibitor (H89) and EPAC activator (8-CPT-2'Me-cAMP) restored migration in Rsu1 knockdown cells (**Figure 15B**). As expected, treatment with an EGF-R inhibitor (AG-1478), PKA-EPAC activator (8-bromo cAMP) or adenylyl cyclase activator (Forskolin) resulted in a decrease in migration (**Figure 15B**).

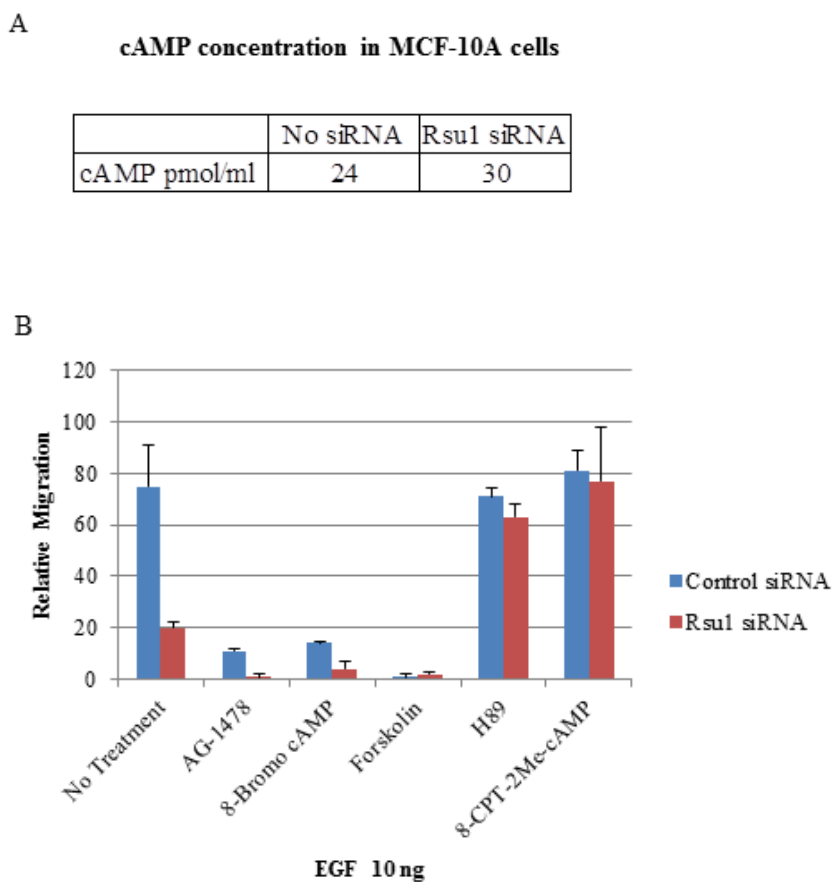


Figure 15. PKA and EPAC: potential targets of Rsu1 signaling. MCF-10A cells were transfected with an Rsu1 siRNA. (A) 72 hours post-transfection cells were replated in the 96 well cAMP ELISA plate. Levels of cAMP were calculated by absorbance readings at 405 nm. (B) 72 hours post-transfection, cells were treated with inhibitors or activators of cAMP signaling for 24 hours. Cells were fixed the following day. Quantitation was performed by staining cells with crystal violet and reading absorbance at 570 nm. The results are displayed as the mean of 4 independent wells \pm SE, * $P \leq 0.005$. 80% of cells were depleted for Rsu1.

Moreover, treatment with a PKA inhibitor (H89) or an EPAC activator (8-CPT-2'-Me-cAMP) resulted in the recovery of FAs and increased spreading in Rsu1 knockdown cells (**Figure 16**). However, actin stress fibers and the association of the FAs proteins with the actin were not fully restored. Together these results suggest a role for Rsu1 in the regulation of PKA and EPAC signaling, and subsequently their downstream effectors, including VASP, cofilin, Rap1 and RIAM.

Rsu1 regulates RACK1 and PDE4D5 localization in MCF-10A cells

Since data from our laboratory revealed a role for Rsu1 in cAMP signaling, interaction of Rsu1 with RACK1 was examined. Co-immunoprecipitation studies showed that immunoprecipitates of Rsu1 from Ras transformed human astrocytes also contained RACK1 (**Figure 17**). However, yeast two hybrid analysis of Rsu1 and RACK1 interaction failed to detect evidence of direct binding (data not shown).

Localization of RACK1 was altered in Rsu1-depleted cells compared to control cells as determined by confocal microscopy (**Figure 18**). In control cells, RACK1 is colocalized with Rsu1 at FA sites (**Figure 18**). In contrast, RACK1 is concentrated around the perinuclear region in Rsu1- or PINCH1-depleted cells (**Figure 18**).

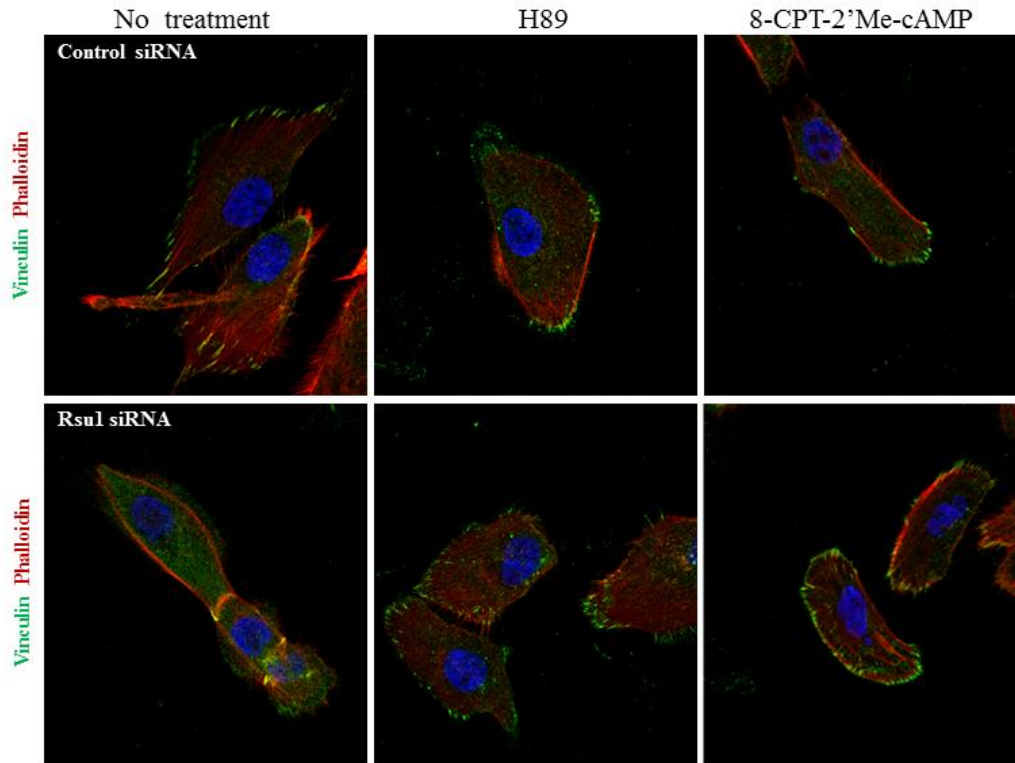


Figure 16. Treatment with H89 or 8-CPT-2'Me-cAMP restored FAs in Rsu1 depleted cells. MCF-10A cells were transfected with a Control and Rsu1 siRNA. (A) 72 hours post-transfection, cells were treated with H89 or 8-CPT-2'Me-cAMP for 24 hours. Cells were fixed and assayed by immunofluorescence the following day. TRITC-Phalloidin and vinculin antibodies were used to identify changes in FAs and actin. Nuclei were counterstained with DAPI. 80% of cells were depleted for Rsu1.

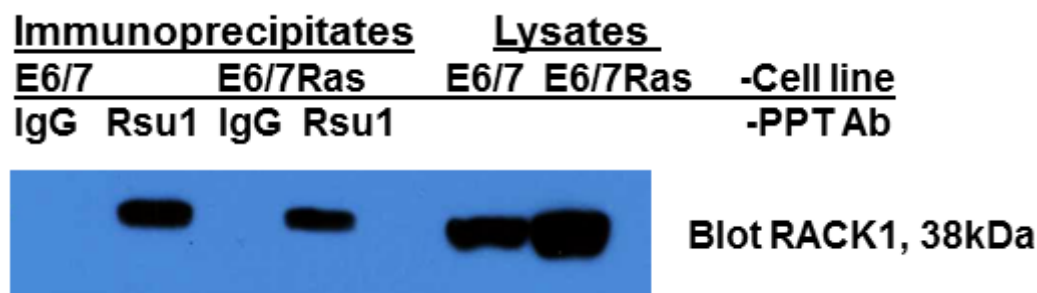


Figure 17. RACK1 co-precipitates with Rsu1. Lysates of immortalized human astrocytes and the Ras transformed astrocyte cell line were immunoprecipitated with anti-Rsu1. The immunoprecipitates were analyzed by Western blotting for co-immunoprecipitation of RACK1.

Furthermore, microscopy studies revealed a change in the proximity of RACK1 and PDE4D5 in Rsu1 or PINCH1-depleted cells compared to the controls (**Figure 19**). In control cells, RACK1 and PDE4D5 are located in close proximity with each other, while in Rsu1 or PINCH1 knockdown cells this association is lost (**Figure 19**). Together these data revealed a role for Rsu1 in cAMP signaling and established the importance of Rsu1 regulation in the localization of RACK1 and PDE4D5.

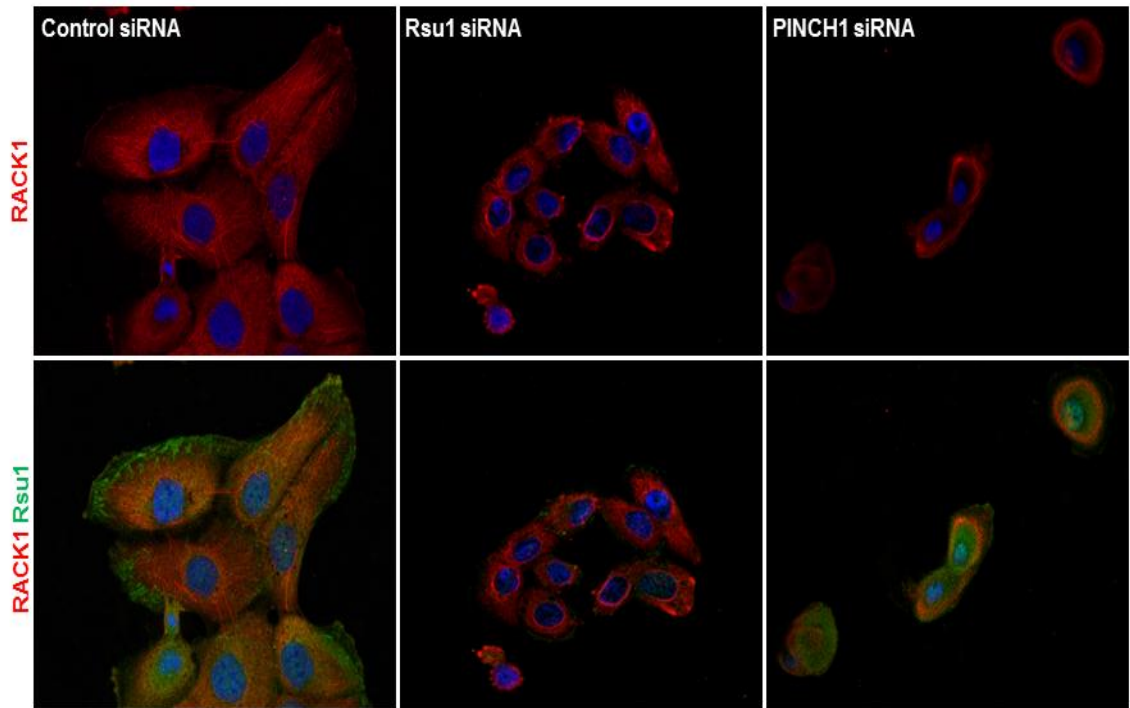


Figure 18. Rsu1 is required for localization of RACK1. MCF-10A cells were transfected with a Control, Rsu1 and PINCH1 siRNA and plated on tissue culture plates and fibronectin coverslips. Coverslips were fixed at 96 hours post-transfection and assayed by immunofluorescence using anti-Rsu1 and anti-RACK1. Nuclei were counterstained with DAPI. 80% of cells were depleted for Rsu1, while 90% were depleted for PINCH1.

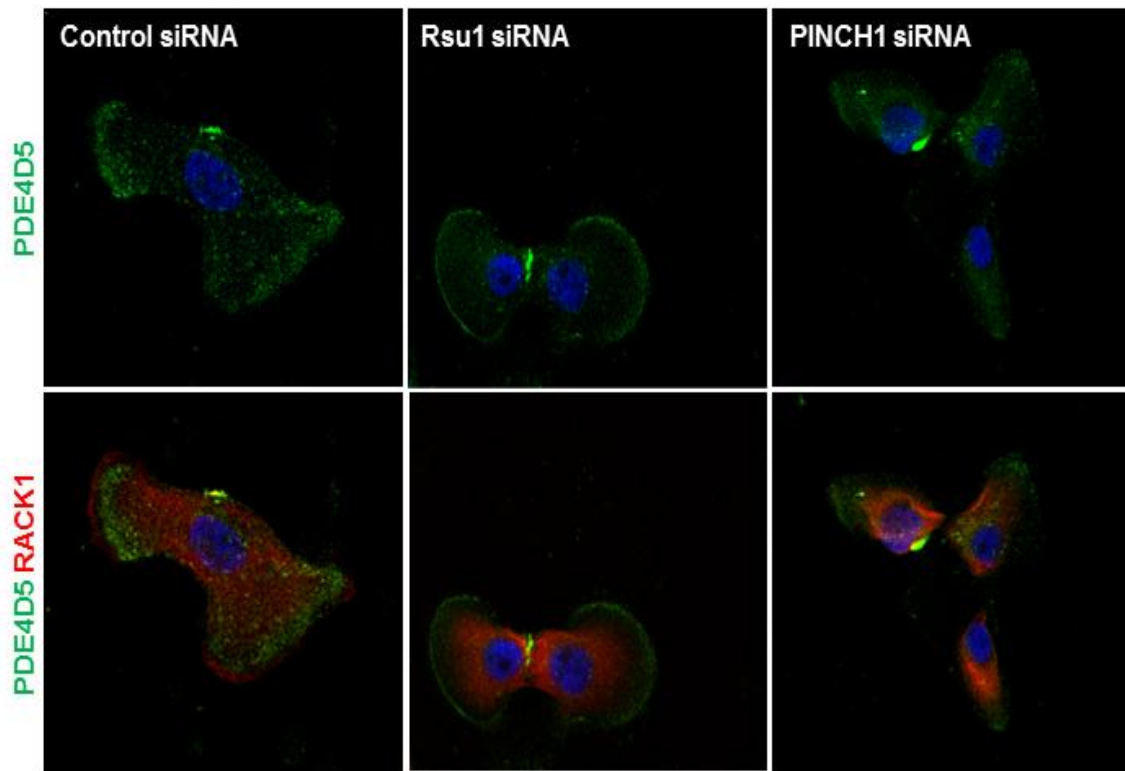


Figure 19. Rsu1 and PINCH1 are required for RACK1 and PDE4D5 localization.

MCF-10A cells were transfected with a Control, Rsu1 and PINCH1 siRNA and plated on tissue culture plates and fibronectin coverslips. Coverslips were fixed at 96 hours post-transfection and assayed by immunofluorescence using anti-PDE4D5 and anti-RACK1. Nuclei were counterstained with DAPI. % of Rsu1 depleted cells was 80%, while 90% of cells were depleted for PINCH1. PDE4D5 was reduced by 36% in Rsu1-depleted cells and by 32% in PINCH1 knockdown cells.

Chapter 4: Discussion

In accordance with the literature, depletion of Rsu1 or PINCH1 resulted in the predicted decrease in cell adhesion and migration of MCF-10A cells. It was also demonstrated that Rsu1-mediated effects were not a result of changes in cell proliferation. In contrast, PINCH1 depleted cells exhibited a dramatic decrease in cell proliferation, but these changes in proliferation in PINCH1 knockdown cells were not due to changes in viability. A role for PINCH1 in cell proliferation has been described, since the knockout of PINCH1 in murine embryos decreased cell proliferation (Liang et al., 2005).

Depletion of Rsu1 caused significant reduction in PINCH1, while PINCH1 depletion resulted in only a modest reduction in Rsu1. This observation is in agreement with other studies that imply a function for Rsu1 in regulating the IPP complex through PINCH1 and suggests a role for Rsu1 in PINCH1 stabilization (Dougherty et al., 2005; Elias et al., 2012; Kadrmas et al., 2004). Differences in cell proliferation between Rsu1 and PINCH1 knockdown cells, as well as the drastic phenotype observed in PINCH1 depleted cells, may be due to the different effects of Rsu1 and PINCH1 depletion on ILK expression. While Rsu1 reduction causes a mild decrease in ILK expression, PINCH1 depletion results in a dramatic decrease of ILK levels. A decrease in ILK translates into a reduction in the number of ILK molecules available for parvin binding. Since parvins have a critical function in the regulation of actin cytoskeleton dynamics and FA turnover, disruption of ILK following PINCH1 depletion may result in a more pronounced defect in adhesion and migration compared to Rsu1 knockdown cells. Although ILK also associates with PINCH1, ILK-Parvin binding is most critical for actin mediated functions in adhesion (Fukuda et al., 2009; Wickstrom et al., 2010b).

Rsu1 or PINCH1 depletion results in altered distribution and localization of the FA proteins Paxillin, Vinculin, Talin and ILK. Furthermore, Rsu1 or PINCH1 knockdown cells lose the ability to form FA sites, which is characteristic of cells undergoing detachment. Since the RIPP complex is an important regulator of integrin signaling, disruption of this complex may impair signals that are required for the formation of FAs. A failure in integrin activation or the transmitting of signals from the integrins to downstream effectors will affect the recruitment of proteins required for the creation of integrin adhesions sites. Changes on talin location and distribution seen in Rsu1 or PINCH1 knockdowns support the idea of a defect in integrin activation. Alternatively, a defect in actin assembly or disassembly would impair the ability of the cells to create structures required for the locomotive force in migrating cells such as filopodia and lamellipodia. Disruption of the actin-myosin interaction which is essential for the translocation of the cell body and retraction could occur. Furthermore, a defect in actin cytoskeleton dynamics would affect FA maturation and subsequently the creation of integrin adhesion sites. The loss of stress fibers along with the increased phosphorylation of actin regulatory proteins such as p-VASP and p-cofilin observed in Rsu1 or PINCH1 knockdown cells supports the idea of a defect in actin cytoskeleton remodeling.

Levels of phosphorylated-FAK (Tyr 397) decreased significantly in Rsu1 and PINCH1 depleted cells. This could be due to either a disruption in integrin or Src activation signaling which prevents autophosphorylation of FAK or an increase in the activity or the levels of protein tyrosine phosphatases. Previous studies demonstrated that while Rsu1 blocked Ras transformation it slightly enhanced the transforming activity of Src (Cutler et al., 1992). Depletion of Rsu1 or PINCH1 may reduce the recruitment of Src

to integrin adhesion sites which subsequently leads to a decrease in phosphorylated FAK. A recent study revealed that FAK activation is required for recruitment of talin to nascent adhesions and FA turnover (Lawson et al., 2012). The inability of FA proteins to migrate to adhesion sites could be influenced by the reduction in the levels of phosphorylated-FAK. Thus, Rsu1 may be regulating migration in MCF-10A cells in part by a FAK mediated mechanism. However, a decrease in the phosphorylation of FAK is also characteristic of non-adherent cells. Thus, the FAK phenotype seen in Rsu1 or PINCH1 depleted cells may simply be a result of cell detachment and not a direct consequence of the absence of Rsu1 or PINCH1.

The level of most FA proteins did not change in response to Rsu1 or PINCH1 knockdown. Although EGF-R expression was decreased, studies conducted in the laboratory confirmed that the receptor was functional and that Rac, Rho and Cdc42 were activated in response to EGF stimulation. A body of evidence implicates EGF-R signaling in regulation of cell migration (Duchek and Rorth, 2001; Katz et al., 2007; Tarcic et al., 2012), and our studies confirmed that EGF signaling was required for cell migration in MCF-10A cells. Since EGF-R was functional in Rsu1 or PINCH1 knockdown cells, these data show that, while deregulation of this receptor could contribute, it is not the sole mechanism by which Rsu1 or PINCH1 regulates adhesion and migration.

β 1 integrin expression was elevated following depletion of Rsu1 or PINCH1. It is our hypothesis that Rsu1 or PINCH1 knockdown cells expressed elevated levels of β 1 integrin either as a compensatory mechanism for the lack of adhesion and migration displayed by these cells or because it was not properly recycled in the knockdown cells.

The localization and distribution of $\beta 1$ integrin in “endosome like structures” and a disorganized caveoli staining expanded the Rsu1 and IPP functions as potential regulators of lipid raft/caveoli formation and endocytic transport. Although there are no reports of any involvement of the IPP complex in integrin recycling, experimental studies had demonstrated a role for ILK in caveola formation and trafficking to the cell membrane (Meyer et al., 2005; Wickstrom et al., 2010a). In adherent cells, integrins prevent lipid raft internalization by sequestering caveolin at the FA sites (Caswell et al., 2009). Conversely, cells undergoing detachment release caveolin from the FA (Caswell et al., 2009), as seen in Rsu1 or PINCH1 depleted cells. A close examination of endosomal markers and recycling assays revealed minor differences in the distribution and localization between control, Rsu1 or PINCH1 depleted cells. As stated above, these differences would be expected in cells undergoing detachment. The effects on caveolin and endosome markers upon Rsu1 or PINCH1 knockdown could be a secondary effect (through decrease in ILK) and not the triggering event for the changes in adhesion and migration.

An increase in migration accompanied by smaller FAs and loss of actin stress fibers was observed upon treatment of MCF-10A cells with a PKA inhibitor (H89). Inhibition of Mek, PI3 Kinase, Rap1, PKC and EGF-R led to the expected decreased in migration. Interestingly, cells treated with PI3 kinase and PKC inhibitors displayed larger adhesion and well-formed stress fibers despite their decreased migration. The decrease in migration upon Rsu1 or PINCH1 knockdown was associated with the loss of FAs. In contrast, studies conducted in FAK-deficient mice revealed a reduction in the mobility of

embryonic cells as a result of an increase in FAs (Ilic et al., 1995). An increase or decrease in FA formation and stability could lead to impaired cell migration.

Cells undergoing detachment expressed elevated levels of cAMP (Howe and Juliano, 2000; Norambuena and Schwartz, 2011). Since Rsu1 depleted cells exhibited an increase in cell detachment, levels of cAMP were determined in Rsu1 knockdown cells. Rsu1 depleted cells expressed higher levels of cAMP, suggesting a role for Rsu1 in cAMP signaling. An increase in cAMP levels may be caused by a defect in phosphodiesterase activity or constitutive stimulation of adenylyl cyclase by the G proteins. Restoration of cell migration in Rsu1 depleted cells was observed upon treatment with a PKA inhibitor or an EPAC activator. However, actin stress fibers were not fully recovered suggesting that perhaps there is (are) other signaling pathway(s) or signaling molecule(s) affected by Rsu1 regulation. Consistent with this result, we demonstrated an increase in the phosphorylation of the PKA downstream effectors VASP and cofilin upon Rsu1 or PINCH1 knockdown. Since there are no reports that associate the RIPP complex with cAMP signaling, these studies provide evidence of an unknown function of Rsu1 and its implications in the regulation of cell migration.

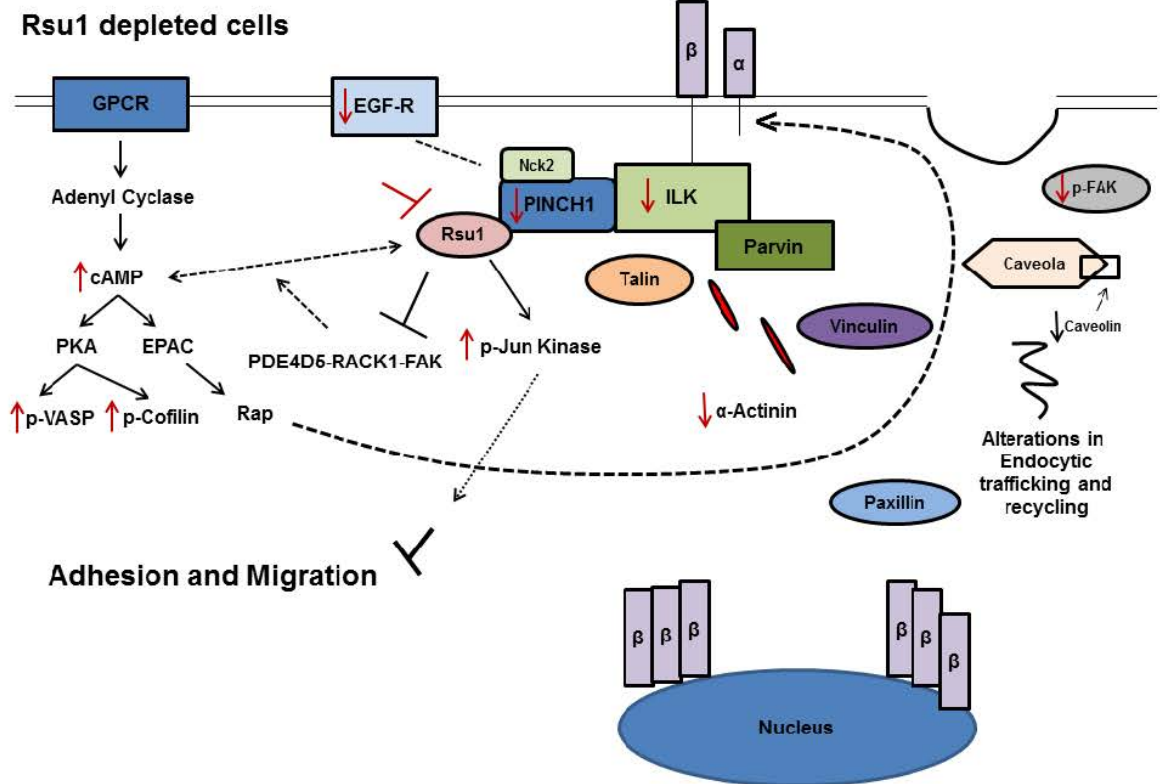
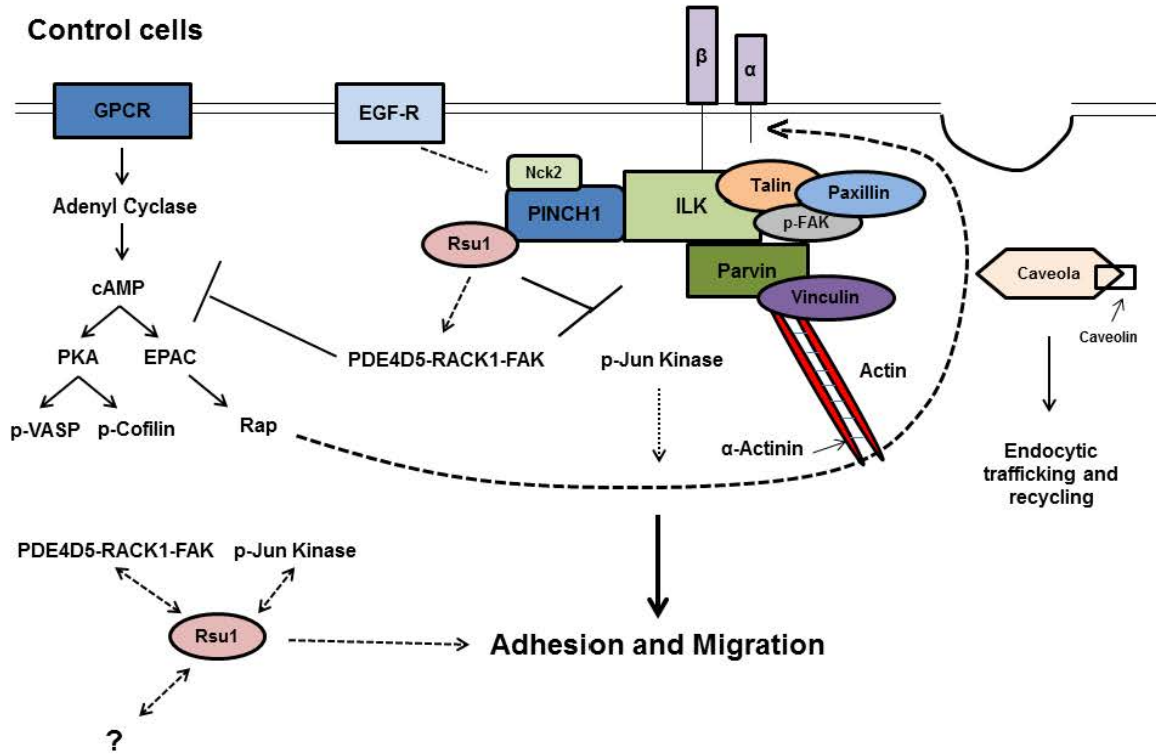
An interaction of Rsu1 with RACK1 was identified in a glioma cell line. A FAK mutant that does not bind to RACK1 resulted in reduced spreading and a change in the localization of RACK1 from adhesion sites to cytoplasmic locations (Serrels et al., 2010). The same phenotype was observed in Rsu1 or PINCH1 depleted cells. Additionally, Rsu1 or PINCH1 inhibition resulted in a slight decrease in PDE4D5 expression. Moreover, RACK1 and PDE4D5 proximity with each other is lost in Rsu1 or PINCH1 depleted cells. It is our hypothesis that Rsu1 has a potential role in the formation, stability or

localization of the FAK-RACK1-PDE4D5 complex. Decreased levels of p-FAK as well as PDE4D5, as a result of Rsu1 depletion, could impair the ability of the complex to form and function. A defect in the complex may result in elevated levels of cAMP, thereby inhibiting the spreading, adherence and migration of the cells.

Reconstitution of Rsu1-depleted cells with an Rsu1 mutant that fails to bind PINCH1 partially restored actin stress fibers, FAs and migration, indicating that binding of Rsu1 to PINCH1 is likely not the sole mechanism by which Rsu1 regulates actin-dependent adhesion and migration. Partial restoration of adhesion sites by the Rsu1 mutant was not due to an increase in PINCH1 or ILK expression suggesting that the Rsu1 non-binding mutant may be restoring adhesion and migration through an IPP independent mechanism. Although, inhibition of one of the members of the IPP complex results in a decrease in expression of the others, it does not lead to complete inhibition (Legate et al., 2006), suggesting a role for these proteins outside IPP signaling. Since Rsu1 inhibits JNK signaling, the Rsu1 mutant may regulate adhesion and migration by altering JNK activation. Rsu1 is required for the viability of *Drosophila* embryos with disrupted PINCH-ILK binding (Elias et al., 2012). Hence, the Rsu1 may also function in survival and apoptosis via regulation of JNK activation during detachment of MCF-10A cells. Alternatively, the Rsu1 non-binding mutant may be exerting its function via the regulation of RACK1. Partial recovery of adhesion sites and stress fibers in Rsu1 depleted cells infected with the Rsu1 mutant could be a result of restored regulation of cAMP signaling by the FAK-RACK1-PDE4D5 complex.

Proposed Model

Rsu1 and PINCH1 regulate cell adhesion and migration by directing FA formation, localization and distribution, as well as assembly and disassembly of the actin cytoskeleton. Our data also revealed a potential function for Rsu1 and the IPP complex in cAMP signaling and identified PKA and EPAC as potential regulators of Rsu1 dependent adhesion and migration. Furthermore, we demonstrated a role for Rsu1 and the IPP complex in the localization of the RACK1-PDE4D5 complex. Rsu1 depletion results in a decrease in the levels of PINCH1 and this is a significant part of Rsu1-mediated detachment. Disruption in cell adhesion and migration upon Rsu1 or PINCH1 knockdown could be explained by deregulation of FAs, lack of stress fibers, changes in the endocytic pathway, increase in β 1 integrin, decreased EGF-R, loss of p-FAK, increase in cAMP levels and phosphorylation of actin binding proteins. Additionally, experiments conducted with an Rsu1 mutant that does not bind to PINCH1 revealed a role for Rsu1 independent from IPP signaling. Rsu1 may be regulating cell adhesion and migration in part through a Jun Kinase or a FAK-RACK1-PDE4D5 mechanism.



Chapter 5: References

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